UNITED STATES
DEPARTMENT OF
COMMERCE
PUBLICATION

NBS TECHNICAL NOTE 587

Organic Chemistry Section:

Summary of Activities, July 1970 to June 1971

U.S.
DEPARTMENT
OF
COMMERCE

National
Bureau
of
Standards

The National Bureau of Standards[1] was established by an act of Congress March 3, 1901. The Bureau's overall goal is to strengthen and advance the Nation's science and technology and facilitate their effective application for public benefit. To this end, the Bureau conducts research and provides: (1) a basis for the Nation's physical measurement system, (2) scientific and technological services for industry and government, (3) a technical basis for equity in trade, and (4) technical services to promote public safety. The Bureau consists of the Institute for Basic Standards, the Institute for Materials Research, the Institute for Applied Technology, the Center for Computer Sciences and Technology, and the Office for Information Programs.

THE INSTITUTE FOR BASIC STANDARDS provides the central basis within the United States of a complete and consistent system of physical measurement; coordinates that system with measurement systems of other nations; and furnishes essential services leading to accurate and uniform physical measurements throughout the Nation's scientific community, industry, and commerce. The Institute consists of a Center for Radiation Research, an Office of Measurement Services and the following divisions:

> Applied Mathematics—Electricity—Heat—Mechanics—Optical Physics—Linac Radiation[2]—Nuclear Radiation[2]—Applied Radiation[2]—Quantum Electronics[3]—Electromagnetics[3]—Time and Frequency[3]—Laboratory Astrophysics[3]—Cryogenics[3].

THE INSTITUTE FOR MATERIALS RESEARCH conducts materials research leading to improved methods of measurement, standards, and data on the properties of well-characterized materials needed by industry, commerce, educational institutions, and Government; provides advisory and research services to other Government agencies; and develops, produces, and distributes standard reference materials. The Institute consists of the Office of Standard Reference Materials and the following divisions:

> Analytical Chemistry—Polymers—Metallurgy—Inorganic Materials—Reactor Radiation—Physical Chemistry.

THE INSTITUTE FOR APPLIED TECHNOLOGY provides technical services to promote the use of available technology and to facilitate technological innovation in industry and Government; cooperates with public and private organizations leading to the development of technological standards (including mandatory safety standards), codes and methods of test; and provides technical advice and services to Government agencies upon request. The Institute also monitors NBS engineering standards activities and provides liaison between NBS and national and international engineering standards bodies. The Institute consists of the following divisions and offices:

> Engineering Standards Services—Weights and Measures—Invention and Innovation—Product Evaluation Technology—Building Research—Electronic Technology—Technical Analysis—Measurement Engineering—Office of Fire Programs.

THE CENTER FOR COMPUTER SCIENCES AND TECHNOLOGY conducts research and provides technical services designed to aid Government agencies in improving cost effectiveness in the conduct of their programs through the selection, acquisition, and effective utilization of automatic data processing equipment; and serves as the principal focus within the executive branch for the development of Federal standards for automatic data processing equipment, techniques, and computer languages. The Center consists of the following offices and divisions:

> Information Processing Standards—Computer Information—Computer Services—Systems Development—Information Processing Technology.

THE OFFICE FOR INFORMATION PROGRAMS promotes optimum dissemination and accessibility of scientific information generated within NBS and other agencies of the Federal Government; promotes the development of the National Standard Reference Data System and a system of information analysis centers dealing with the broader aspects of the National Measurement System; provides appropriate services to ensure that the NBS staff has optimum accessibility to the scientific information of the world, and directs the public information activities of the Bureau. The Office consists of the following organizational units:

> Office of Standard Reference Data—Office of Technical Information and Publications—Library—Office of International Relations.

---

[1] Headquarters and Laboratories at Gaithersburg, Maryland, unless otherwise noted; mailing address Washington, D.C. 20234.
[2] Part of the Center for Radiation Research.
[3] Located at Boulder, Colorado 80302.

Organic Chemistry Section:
Summary of Activities, July 1970 to June 1971

Robert Schaffer, Editor

Organic Chemistry Section
Analytical Chemistry Division
Institute for Materials Research
National Bureau of Standards
Washington, D.C. 20234

National Bureau of Standards Technical Note 587

FOREWORD

The Analytical Chemistry Division was established as a separate division at the National Bureau of Standards on September 1, 1963, and became part of the Institute for Materials Research in the February 1, 1964, reorganization. It consists at present of nine sections and about 100 technical personnel encompassing some 60 different analytical competences from activation analysis and atomic absorption to vacuum fusion and x-ray spectroscopy. These competences, and in turn the sections which they comprise, are charged with research at the forefront of analysis as well as awareness of the practical sample, be it standard reference material or service analysis. In addition it is their responsibility to inform others of their efforts.

Formal publication in scientific periodicals is a highly important output of our laboratories. In addition, however, it has been our experience that informal, annual summaries of progress describing efforts of the past year can be very valuable in disseminating information about our programs. A word is perhaps in order about the philosophy of these yearly progress reports. In any research program a large amount of information is obtained and techniques developed which never find their way into the literature. This includes the "negative results" which are so disappointing and unspectacular but which can often save others considerable work. Of importance also are the numerous small items which are often explored in a few days and which are not important enough to warrant publication— yet can be of great interest and use to specialists in a given area. Finally, there are the experimental techniques and procedures, the designs and modifications of equipment, etc., which often require months to perfect and yet all too often must be covered in only a line or two of a journal article.

Thus our progress reports endeavor to present this information which we have struggled to obtain and which we feel might be of some help to others. Certain areas which it appears will not be treated fully in regular publications are considered in some detail here. Other results which are being written up for publication in the journal literature are covered in a much more abbreviated form.

At the National Bureau of Standards, publications such as these fit logically into the category of a Technical Note. We plan to issue these summaries for all our sections. The following is the seventh annual report on progress of the Organic Chemistry Section.

W. Wayne Meinke, Chief
Analytical Chemistry Division

PREFACE

This is the seventh annual progress report of the Organic Chemistry Section of the Analytical Chemistry Division, and it describes the activities of the Section from July 1, 1970 to June 30, 1971. Unlike the formal, journal publications that emanate from the Section from time to time, this report summarizes the activities of the entire Organic Chemistry Section during that period.

Following the established pattern within this Division the programs of the Section have both a research and a sample aspect: about half of the total effort of the Section has been applied to research, and the remainder to Standard Reference Materials (SRMs). The Section must concern itself with many subjects in organic chemistry in supporting the National Bureau of Standards' overall mission, which is to contribute, where possible, to the solution of nationwide problems through the enhancement of meaningful measurement. To that end, in the past year, much of the Section's effort was directed to developing SRMs needed in clinical chemistry laboratories. This has entailed working with organic compounds that fall into a number of classical areas of organic chemistry. To maintain overall unity in the Section's program, considerable attention is given to one major area of organic chemistry, the carbohydrates, developing therein the Section's competences in organic analysis, characterization, and synthesis. These competences are utilized to the maximum extent for the development of SRMs. Our efforts in serving clinical chemistry have been fostered by the National Institute of General Medical Services of the National Institutes of Health.

In this report, in order to describe procedures adequately, it has occasionally been necessary to identify commercial equipment. In no case does such identification imply NBS recommendation or endorsement, nor does it imply that the equipment identified is necessarily the best available for the purpose.

Robert Schaffer, Chief
Organic Chemistry Section

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ORGANIC CHEMISTRY SECTION: SUMMARY OF ACTIVITIES,
JULY 1970 TO JUNE 1971
Edited by Robert Schaffer

This report of the Organic Chemistry Section of the
National Bureau of Standard's Institute for Materials
Research provides a summary of a year's scientific activity;
as such, it covers both work that was completed and some
that is still in progress. The development of Standard
Reference Materials (SRMs) and their certification, and
journal publications describing aspects of that work, as
well as other research activities, are the output of the
Section.

In the SRM category, the major subjects of this report
are bilirubin, cholesterol, cortisol, D-glucose, 4-hydroxy-
3-methoxy-DL-mandelic acid (VMA), D-mannitol, and the reduced
form of nicotinamide adenine dinucleotide (NADH).

Studies concerning carbohydrates include synthetic
work with D-psicose, n.m.r. employing internuclear double-
resonance, and mass spectroscopy of 6-deoxy-1,2:3,5-di-O-
isopropylidene-α-D-glucose, spectroscopic studies of
derivatives of 6-amino-6-deoxy-D-glucose-6-^{15}N (employing
mass spectrometry, Fourier-transform, ^{13}C-n.m.r. and hetero-
nuclear, ^{15}N indor techniques), and p-bromophenylosotri-
azoles of inositols.

Other research utilized e.s.r. and involved periodic
acid oxidation of azines, solid-state oxidation of
benzenehexol on active manganese dioxide, and the formation
of stable radical-anions from glyoxal bis(phenylhydrazone).

Key words: Bilirubin; cholesterol; e.s.r.; D-mannitol;
NADH; n.m.r.; purity; Standard Reference Materials; SRM; VMA.

1. CLINICAL STANDARDS

A. Bilirubin

1. A Crystalline Bilirubin SRM for Clinical Analysis

Improved analyses for bilirubin in blood are being
pursued by Standards Committees of the American Association
of Clinical Chemists, the College of American Pathologists,
the National Committee for Clinical Laboratory Standards,
and the International Federation of Clinical Chemists. The
National Bureau of Standards, as a part of its effort with
the overall problem, has developed a supply of bilirubin
as a Standard Reference Material (SRM).

In 1962, in the absence of a method for characteri-
zing the purity of bilirubin preparations, it was recommended
that the crystalline bilirubin used for preparing needed
standard solutions [1] should show an absorptivity (±2 SD)
of 60,700 ±1,600 liter·cm^{-1}·mol^{-1} at 453 nm in chloroform
at 25 °C. Bilirubin can be purified to meet this requirement
by the procedure of Henry, Jacobs, and Chiamori [2]. Neither
a modification of that procedure by Fog [3] nor other spectro-
photometric measurements on preparations reported since [4]
suggest that the absorptivity of bilirubin might actually
exceed the value recommended.

After crystallizing bilirubin preparations, it is common
practice to remove adhering solvent by a vacuum treatment
at room temperature in the presence of a desiccant. We
found some commercial bilirubin preparations having low
spectrophotometric absorptivities would lose weight on
drying, but losses were insufficient to improve their molar
absorptivities materially; samples having high absorptivity
lost very little weight [2]. Work at NBS has shown that
bilirubin crystallized from chloroform retains a significant
proportion of this solvent which is not easily volatilized.
For example, on heating a high-absorptivity sample at 110 °C
for 9 h at 0.3 torr, only a slight weight-loss resulted

2

($\sim$0.1%), even though by analysis this material was found to contain chloroform in excess of 1.5%.

The SRM bilirubin contains 0.8% of chloroform which needs 4 h at 250 °C and 0.3 torr to be volatilized. (Such heating destroys the sample.) At 180 °C, with like amounts of time and vacuum, only about half of the chloroform is removed. Numerous drying experiments and chloride determinations showed that the chloroform was homogeneously distributed in the SRM. G.l.c. [5] and n.m.r. measurements assured that chloroform was present and was being volatilized.

In the purification process of Henry et al. [2], brown pigment is removed from a chloroform solution of bilirubin by extracting the solution with 5% aqueous sodium bicarbonate. We have quantitated the extraction procedure by determining the weights of (a) the unextracted and (b) the bicarbonate-extracted material, which contains little bilirubin. The latter is recovered by neutralizing the bicarbonate extracts and extracting with chloroform. The absorbance maximum of these chloroform extracts of recovered bilirubin occurs usually at $\sim$449 nm. The weight of material in the extract is determined by drying. The overall recovery of material is almost quantitative. By this technique, some commercial preparations showed upwards of 10% of extractable impurity; the better of the commonly available bilirubin preparations we examined contained only as little as 2–3%. The SRM has none; see table 1.

The values cited in the literature for the molar absorptivity of bilirubin in chloroform were calculated on the assumption that the measurements had been made on 100%-pure materials. Judged on that basis for comparison, the SRM bilirubin (as supplied) shows (measurements by Dr. R. W. Burke and E. R. Deardorff of Section 310.04) a molar absorptivity (±2 SD) of 61,100 ±400 liter·cm^{-1}·mol^{-1} at 453 nm.

3

Table 1. Some data showing levels of impurity extractable
with 5% aqueous sodium bicarbonate from samples
of bilirubin dissolved in chloroform

| | | Recovery | |
Mfr.'s lot no.	Sample (mg)	Unextracted (mg)	Extracted[a] (mg)
5310	107.20	95.10	11.88
39148A	99.63	90.65	9.39
7322	106.97	100.35	6.85
8162	105.54	102.88	2.48
SRM	95.61	95.34	0.23

[a]Weights include about ∿0.2 mg of bilirubin.

2. Pink-fluorescing Impurity (366-nm Irradiation)
Present in Bilirubins
a. Commercial Bilirubins

(A. J. Fatiadi)

In May 1968, by use of thin-layer chromatography (t.l.c.)
[activated Silica Gel G overloaded as a spot with bilirubin
(∿50 µg) and developed with 8:1:1 (v/v) benzene–N,N-dimethyl-
formamide–acetic acid], we observed with bilirubin samples
from four different commercial suppliers an impurity that
fluoresced under 366-nm light. Such thin-layer chromatograms
showed (a) at the origin, a yellow-orange spot (non-migrated
bilirubin), (b) extending from the origin, a faint yellow-
orange streak (bilirubin), (c) at R_f 0.64, a green spot
(presumed due to biliverdine), (d) at R_f 0.81, a faintly
visible, yellow spot that fluoresced under 366-nm irradiation,
and (e) a yellow-orange spot at R_f 0.86 (bilirubin). The
bilirubin spots were identified by isolation and spectrophoto-
metry, but the fluorescent material could not be evaluated.

4

However, further attention was given to the fluorescent
component after the presence of a pink-fluorescing impurity
in SRM bilirubin was reported to us by Drs. D. C. Nicholson
and M. Stoll [5], who used t.l.c. on polyamide plates, with
3:1 (v/v) methanol—aqueous ammonium hydroxide (3.3%) as the
developer [6]. At about that time, we had found that, by
oxidation of bilirubin with periodic acid, there resulted a
biliverdine—iodine charge-transfer complex, and that reduction
of the isolated material with sodium borohydride regenerated
bilirubin [7]. This product was found to be free of fluo-
rescent impurity by this t.l.c.; hence, this fluorescence
was not an artifact of the chromatography.

b. SRM Bilirubin

(B. F. West and A. Cohen)

On a polyamide thin-layer plate heavily loaded as a
spot with SRM bilirubin ($\sim$75 µg) and developed with the
methanol—aqueous ammonium hydroxide, the bilirubin was found
as an elongated spot at R_f $\sim$0.57, and a minor, second
component was found at R_f 0.74. The latter was barely
visible as a yellow spot, but, when illuminated with 366-nm
light for about 2 min, a somewhat stable, pink fluorescence
developed in its place, and after this exposure to uv, the
color of this spot under ordinary light was found changed to
a very pale gray. Two-dimensional chromatography did not
separate a second pink-fluorescing material from the bili-
rubin.

The fluorescent component in SRM bilirubin was also seen
by t.l.c. on cellulose. On either commercially precoated
plates or hand-spread layers of microcrystalline cellulose,
development with methanol, which did not move the bilirubin
from the origin, separated a component having pink fluo-
rescence (under 366-nm irradiation). It was found near the
solvent front. Its fluorescence is visible immediately on
exposure to uv light, and persists with further uv

5

Certificate of Analysis
Standard Reference Material 916
Bilirubin

This standard reference material is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of procedures employed in clinical analysis and for routine critical evaluation of the daily working standards used in these procedures.

Purity (bilirubin content) . . . . 99.0 percent
Chloroform . . . . 0.8 percent
Insoluble (in chloroform) . . . . .less than 0.01 percent
Ash . . . . 0.01 percent

The value of the bilirubin content has a possible estimated inaccuracy of two percent. (See section on thin-layer chromatography).

The material in chloroform (ACS reagent containing ethanol) at 25.0 °C shows at 453 nm an absorptivity (± 1 SD) of 104.6 ± 0.2 liter cm^{-1} g^{-1}. In methyl sulfoxide at 25.0 °C, the absorptivity (± 1 SD) of the material at 453 nm is 107.7 ± 0.6 liter cm^{-1} g^{-1}.

It is recommended that bilirubin and its solutions be handled only under low-intensity incandescent light. The bilirubin should be stored under conditions that totally exclude light. Once removed from the ampoule, the vial should be replaced for storage in an air-tight container and stored at low temperature. The vial must be allowed to return to room temperature before opening.

The bilirubin used for this standard reference material was obtained from Pfanstiehl Laboratories, Inc., of Waukegan, Illinois. Analyses were performed by R. F. Brady, Jr., R. W. Burke, A. Cohen, B. Coxon, W. D. Dorko, D. Enagonio, A. J. Fatiadi, D. H. Freeman, J. M. Ives, B. A. Johnson, E. C. Kuehner, R. A. Paulson, T. C. Rains, R. Schaffer, W. P. Schmidt, J. T. Sterling, J. K. Taylor, B. F. West, W. L. Zielinski, Jr.

The overall direction and coordination of technical measurements leading to the certification were under the chairmanship of R. Schaffer.

The technical and support aspects concerning the preparation, certification, and issuance of this standard reference material were coordinated through the Office of Standard Reference Materials by T. W. Mears.

Washington, D. C. 20234
March 10, 1971

J. Paul Cali, Chief
Office of Standard Reference Materials

(over)

6

The standard reference material was prepared from material that was isolated from hog bile and crystallized as the acid. It was purified further by treatment in chloroform solution with sodium sulfate according to Fog [1] and recrystallization from chloroform.

Ash was determined on 100-mg samples heated overnight at 250 °C and then for 3 hrs at 650 °C. Flame photometry and atomic absorption measurements on a solution of the ash obtained from 0.275 g of the material showed, in $\mu g/g$ (ppm) of metal: <0.05 lithium, 7.3 sodium, 1.6 potassium, 2.1 calcium, and 2.3 magnesium.

Elemental composition found for the material was: C, 67.36; H, 6.24; N, 9.42; Cl, 0.73. Required for a bilirubin preparation containing 0.8 percent of chloroform: C, 67.25; H, 6.17; N, 9.50; Cl, 0.71. In these analyses, nitrogen was determined by the Kjeldahl technique; and chlorine was determined by Carius digestion followed by gravimetric determination as silver chloride. By the latter method and recalculated as chloroform, the average (± 1 SD) of ten 25-mg samples, was 0.83 ± 0.04 percent. The chloroform content was also obtained by heating at 250 °C at 0.5 torr for 4 hrs; the average (± 1 SD) of six 100-mg samples was 0.79 ± 0.02 percent. (Only about one-half or one-tenth of the chloroform was removed at 180 °C and at 110 °C, respectively.) During gradual heating of the material in a quadrupole mass spectrometer, only ion-fragments characteristic of chloroform were observed until the sample was heated above 300 °C, whereupon water and carbon dioxide were detected, and at a somewhat higher temperature, the mass spectrum typical of bilirubin was obtained. Gas chromatography and nuclear magnetic resonance spectroscopy with the material dissolved in methyl sulfoxide confirmed the presence of chloroform Because the chloroform is so firmly retained and homogeneously dispersed, it is recommended that the material be used as supplied.

Thermogravimetric analysis with the sample heated in a dry nitrogen atmosphere at 5 °C/min showed the initiation of the loss of a large proportion of sample weight at temperatures between 319 and 323 °C; determined in air, however, comparable weight-loss began between 288 and 292 °C.

For determining the absorptivity of the standard material, absorbances were recorded with a Cary 16 spectrophotometer on solutions using a capped 1.000 ± 0.001-cm path-length cuvette in a cell-holder thermostated at 25.0 ± 0.1 °C. Solution preparation and handling were performed under low intensity, incandescent light; at other times, solutions were totally protected from light. To preclude any change in concentration of chloroform solutions due to evaporation of the solvent during solution transfer, nitrogen gas was used to force the solution from the flask through Teflon tubing into the cuvette and, to effect rinsing of the cuvette, from the latter to an overflow. Solutions in methyl sulfoxide were transferred to the cuvette by pipet.

For the determination of chloroform-insoluble matter, sample (0.1 g) and solvent (200 ml), protected from light, were agitated in an ultrasonic bath, heated to 60 °C briefly, and then cooled and filtered, using pressure, (with 50 ml of the solvent for washing) through a tared 0.4 μm-porosity membrane.

Extraction by Bicarbonate. Yellow extracts were obtained by shaking a solution of the standard reference material (0.010 g) in chloroform (40 ml) with 10-ml volumes of 0.6 molar aqueous sodium bicarbonate. Their absorption spectra, with maxima in the range 415--430 nm, showed absorbance values that ranged from 0.035 to 0.030 in successive extracts using 1-cm cells. The qualitative and quantitative compositions of such extracts were examined using the filtered chloroform solutions containing 0.1-g quantities of the standard reference material that were prepared in the tests for chloroform insolubles: Extractions twice with 50-ml volumes of the bicarbonate solution and with water, and neutralization of the combined aqueous extracts followed by extraction into chloroform, yielded pigment (dry weight approximately 0.2 mg) that was shown by its spectrum and specific absorptivity to be typical of bilirubin in chloroform. Thus, bicarbonate extraction provided no evidence of impurities more acidic than bilirubin.

Extraction by Carbonate. Extraction of a 200-ml chloroform solution containing 0.1 g of standard with 100-ml volumes of 0.1 molar sodium carbonate reduced the 1-cm absorbance value of the chloroform layer to approximately 0.22 after the second extraction, and to 0.08 and then 0.01 with the third and fourth extractions, respectively. On washing with water and then evaporating the extracted chloroform layer to dryness, residue weights were typically about 0.01 mg. Hence, this extraction process showed a negligible proportion of nonacidic impurities.

Thin-layer Chromatography. An impurity in the standard material may be detected by thin-layer chromatography with polyamide as the absorbent and 3:1 (v/v) methanol--aqueous ammonium hydroxide (3.3 percent) as the developer [2]. Thus, 0.05 mg of the material (spotted from a chloroform solution) provides, on development, an elongated, yellow-orange streak preceded (and usually separate) by a faintly visible, yellow spot. Under 366-nm irradiation, a pink fluorescence develops very quickly at the location of this spot. The material responsible for the pink fluorescence and its precursor have not been characterized. A means for obtaining a reliable estimate of this contribution to overall impurity has not been ascertained, but is under study. Repurification of the standard material does not affect this behavior.

References

1 J. Fog, *Scand. J. Clin. Lab. Invest.*, **16**, 49 (1964).
2 Z. J. Petryka and C. J. Watson, *J. Chromatog.*, **37**, 76 (1968).

exposure. After uv exposure, the spot exhibits a purple-to-mauve color when viewed under ordinary light. Triple development in the same direction with methanol (but each to a distance 1 cm less than before so that a new solvent front can be distinguished after each development) revealed by the second development a much diminished quantity of separated pink-fluorescing component, and, by the third development, essentially none. (The chromatogram is irradiated only after the third development.)

Evidence for a green pigment, as was found by use of t.l.c. on silica gel was not observed by t.l.c. on either the polyamide or the cellulose.

3. Attempted Isolation of the Pink-fluorescing
   Impurity from Commercial Bilirubin by Preparative
   T.L.C. on Cellulose

(A. J. Fatiadi)

Microcrystalline cellulose (50 g) was stirred in 5:1 (v/v) methanol—chloroform (200 ml) at room temperature for 30 min, filtered, and washed with methanol (100 ml); after it had dried at room temperature, the material was used to prepare a thin-layer plate (20 X 20 cm X 2 mm). The plate was dried for 2 h at room temperature.

Under dim incandescent light, a solution of bilirubin (50 mg) in a mixture of chloroform (1 ml) and diethylamine (0.1 ml) was transferred to the t.l.c. plate as a long streak (nitrogen flushing) about 2 cm from an edge. Chromatography was begun with methanol used as developer, but, as soon as the orange-red band had moved about 3 cm from the origin, the plate was removed from the developer, sprayed with 1% acetic acid in methanol (to convert the complex into free bilirubin), and dried (nitrogen flushing). Then the chromatogram was developed further with methanol. Development was stopped when the solvent front had moved to within 1 cm of

top (45--65 min). After drying (nitrogen flushing), the slightly yellowish band near the top of the plate was scraped off, and extracted with methanol. The extract was concentrated, and the residue (∿0.8 mg) showed, on cellulose t.l.c., a distinct pink fluorescence (366-nm irradiation).

The orange band, on extraction with chloroform and concentration of the extract, gave bilirubin (28 mg) almost free from the pink impurity (t.l.c. on cellulose).

The experiment was repeated with 200 mg of bilirubin on a t.l.c. plate of similar dimensions. The light-yellow band was scraped off, and extracted with methanol (75 ml). Rechromatography of this extract on a cellulose plate (5 cm X 20 cm X 2 mm), followed by extraction with methanol, and concentration, gave a residue (∿1.2 mg) containing much pink-fluorescing impurity (t.l.c. on cellulose).

4. Attempted Isolation of the Pink-fluorescing Impurity by Column Chromatography

(B. F. West)

To the top of a column of methanol-washed, microcrystalline cellulose (30 X 3 cm) protected from light was added a methanolic slurry of microcrystalline cellulose (10 g) that had been coated with SRM bilirubin (90 mg) by evaporating a solution of the bilirubin in chloroform on it. Fractions of effluent were collected using methanol as the developer, and they were monitored for the impurity by t.l.c. on cellulose. When partly concentrated, each of the first six 100-ml fractions of effluent gave similar uv--visible spectra: a broad maximum at 448--450 nm, a sharp doublet at 275--285 nm, and a strong maximum (cut off) at 224 nm. Further concentration of the methanolic solutions led to the deposition of bilirubin. Total concentration followed by extraction of the residue with methanol and filtration gave a green solution, the spectrum of which showed a weak band at

10

650 nm but none at 370 nm. (For biliverdine, the 370-nm band is stronger than that at 650 nm.) A strong peak was now found at 435 nm, and the doublet at 275–285 nm and the peak (cut-off) at 225 nm were still present. There was no absorption between 550 and 600 nm.

Although we carried out these procedures under dim, incandescent illumination in attempts to isolate and quantitate the fluorescing component in the SRM bilirubin, our efforts were hampered by the instability of the substance. As an alternative, we turned to attempts to prepare a quantity of bilirubin that would be free from this impurity, so that we might compare its properties with those of the SRM bilirubin.

5. Preparation of a Reference Sample of Bilirubin; Removal of the Pink-fluorescing Impurity

a. Comments on Alkylamine Complexes of Bilirubin

(R. S. Tipson and A. J. Fatiadi)

We observed that complexes of bilirubin with secondary and tertiary alkylamines are formed in chloroform. This ability of bilirubin to form adducts with alkylamines enabled us to prepare concentrated solutions of bilirubin in very small volumes of chloroform. The structures of these alkylamine complexes have not yet been thoroughly studied; however, their solubility in chloroform or dichloromethane suggests that they are of the acceptor—donor type. Other solvents were found useful for preparing complexes with alkylamines: N,N-dimethylformamide (DMF), N-methylpyrrolid-inone, N,N-dimethylacetamide, formamide, hexamethylphos-phoramide and methyl sulfoxide. Of these, N,N-dimethyl-acetamide was found very efficient, and next best were N-methylpyrrolidinone, and hexamethylphosphoramide. Formamide containing triethylamine dissolves bilirubin very effectively, but, on attempting crystallization, appreciable oxidation of the bilirubin occurs; the same

11

was found with methyl sulfoxide. Ethylenediamine in DMF
dissolves much bilirubin (only a trace of this strong organic
base was needed); however, no crystals of bilirubin were
recovered after prolonged concentration (120 h) of
solutions in a vacuum desiccator.

b. Preparation of Bilirubin—Diethylamine Complex

(A. J. Fatiadi)

Commercial bilirubin (200 mg) was suspended in
chloroform (1 ml) and, while being stirred, the suspension
was treated with diethylamine (0.2—0.3 ml) until all of the
solid had dissolved; sometimes, slight warming at 45 °C
for 2 to 5 sec was necessary. The deep-red solution was
then cooled in an ice-bath, and ethyl ether was added
dropwise to faint turbidity (3 to 4 ml). Dense,
brown-orange, micro-crystalline material separated after
5 to 10 min of additional stirring in the ice-bath. The
product was filtered off, washed with 1:1 (v/v) chloroform-
ethyl ether, and then with ether, and dried in a vacuum
desiccator, to give crop I (55 mg), and, after concentration
and cooling, crop II (85—88 mg).

The complex was quite soluble in chloroform,
dichloromethane, and water, but sparingly soluble in carbon
tetrachloride or ethyl ether. Slight warming of the
complex in ethyl ether (water bath) caused dissociation,
and yielded orange-colored bilirubin. Acidification (acetic
acid or hydrochloric acid) of an aqueous solution of the
complex regenerated bilirubin in high yield. The complex is,
however, very difficult to purify by recrystallization;
usually, recrystallization was performed at room temperature
by dissolution of the complex in a minimum volume of
chloroform containing a small proportion of diethylamine,
followed by precipitation of the complex by addition of
ethyl ether or ethyl ether—heptane. Its elemental composi-

tion conformed closely to that of a 1:1 complex, but the proportions are not ideal.

Anal. Calc. for $C_{33}H_{36}N_4O_6 \cdot C_4H_{10}N$: C, 67.6; H, 7.2; N, 10.6 Found: C, 66.6; H, 7.8; N, 10.7.

T.l.c. on cellulose [methanol or 1:1 (v/v) methanol—acetone] revealed the presence of a second component, possibly the 1:2 complex of bilirubin with diethylamine. The visible spectrum in chloroform ($\lambda_{max}^{CHCl_3}$ 453 nm) corresponded with that of bilirubin; the u.v. and visible spectra in water [$\lambda_{max}^{H_2O}$ 224 (sh), 280, and 435 nm] were typical of a bilirubinate salt.

c. Preparation of the Bis(triethylamine) Salt of Bilirubin

(R. S. Tipson)

Triethylamine (2.0 ml/g) was added to a suspension of bilirubin in N,N-dimethylformamide (20 ml/g), with stirring; addition of an excess of triethylamine (a further 5 ml/g) caused precipitation of a yellow compound. This was filtered off, washed successively with DMF and dry ether, and dried. Its elementary analysis agreed with that calculated for $C_{33}H_{36}N_4O_6 \cdot 2C_6H_{15}N$, and its infrared spectrum (potassium bromide pellet) differed from that of bilirubin.

The salt is not very soluble in methanol, ethanol, acetone, ethyl acetate, dichloromethane, or benzene, but is fairly soluble in chloroform. It is readily soluble in water to give concentrated solutions; on preparation of dilute solutions, dissociation occurs and part of the bilirubin separates out, but addition of a trace of triethylamine causes it to redissolve. B. A. Johnson found that a solution of the salt (0.6 mg) in water (1 ml) had pH 8.83; on standing, the clear solution became cloudy.

d. Growth of Crystals of Bilirubin

(R. S. Tipson)

By dropwise addition of triethylamine (∿2.5 ml/g) to
a stirred suspension of bilirubin in DMF (80 ml/g) until a
clear, brown solution is obtained, followed by slow
evaporation in a vacuum desiccator at 25 °C and 25 torr,
discrete crystals of bilirubin are readily obtained. The
red crystals were examined by C. P. Saylor, who described
them as "blades" (platelets), parallelogram-shaped, about
0.25 X 0.05 mm. Single crystals are pale brown, but a mass
of the crystals is bright carmine red. By slower
evaporation, larger crystals, suitable for X-ray crystal
study, could undoubtedly be grown.

e. Purification of Bilirubin by Recrystallization

(R. S. Tipson)

A solution of bilirubin in DMF containing triethylamine
was prepared; although it appeared to be perfectly clear,
it was (as a precautionary measure) filtered by gravity
through hard filter-paper. The filtrate was evaporated in
a dish in a vacuum desiccator at 25 °C and 25 torr for 1 h,
and then at 25 °C and 1 torr. Crystals grew on the bottom and
sides of the dish, and a dark-green crust formed at the top of
the crystal layer on the walls. Without disturbing the green
crust, the suspension of red crystals was removed, by means
of a bulb pipet fitted with a rubber bulb, and filtered with
suction. The crystals were successively washed with
DMF, 1:1 (v/v) DMF—water, water, and absolute ethanol,
and dried in a vacuum desiccator at 25 °C and 0.01 torr.
Mass-spectrometric examination (by E. E. Hughes) showed
that the crystals were devoid of chloroform (a contaminant
in the original bilirubin), $\underline{N},\underline{N}$-dimethylformamide, or
triethylamine. The infrared spectrum was identical with
that of the original bilirubin. T.l.c. on polyamide with

14

9:2:1 (v/v) methanol—10% ammonia—water (by B. F. West) showed that it contained very much less of the pink-fluorescing impurity than did the original sample of bilirubin.

In a second recrystallization, no green crust formed, indicating that the green component was an impurity, or was derived from an impurity, in the original bilirubin. Twice recrystallized in this way, the bilirubin contained none of the pink-fluorescing material (t.l.c.).

Bicarbonate extracts of a chloroform solution of this bilirubin showed 0.04 absorbance units, whereas SRM bilirubin showed 0.03 (by R. F. Brady, Jr.). The $\varepsilon_{m\underline{M}}$ of this bilirubin in chloroform was 60.7.

f. Procedure for Removal of the Fluorescent Impurity and Other Impurities from Commercial Bilirubin

(A. J. Fatiadi)

In this procedure, two alkylamines, namely, triethylamine and diethylamine, are used in the purifying of bilirubin from its fluorescence-forming impurity. Redissolution of the purified bilirubin in a specified amount of N,N-dimethylformamide (DMF) and triethylamine (NEt_3) can be a troublesome operation, because of the ease of formation of the crystalline adduct. The addition of diethylamine (Et_2NH) overcomes this difficulty. The purification involving the recrystallization of bilirubin from DMF—NEt_3 removes a considerable proportion of the fluorescent impurity, and repetition lowers it to a negligible proportion. However, the recrystallized material still contains other impurities. Hence, the following procedure for the purification of bilirubin includes crystallizations with DMF—NEt_3—Et_2NH, extraction with 5% aqueous bicarbonate [2] and, finally, column chromatography on sodium sulfate [3].

15

Commercial bilirubin (500 mg) was suspended in DMF (10 ml) and stirred magnetically (3 min) to give a homogeneous suspension. About 0.5 ml of NEt_3 was introduced rapidly (30 sec), and the suspension was stirred for about 3 min. Then, slowly (2 to 3 min), more NEt_3 was added. There was a change in color, and a gradual disappearance of the solid. With the further addition of 0.4 to 0.5 ml of NEt_3, a deep, orange-red solution resulted. The solution was filtered (either through glass wool or through "rapid" filter paper), and the filtrate was kept in a porcelain dish over concentrated sulfuric acid in a vacuum desiccator at 10 torr. After 36 h, the deep-red crystals of bilirubin that had formed were carefully filtered (avoiding removal of the brown-green impurity in the crust), and washed successively with DMF and ether; yield 360—380 mg.

Once-recrystallized bilirubin (500 mg) in 10 ml of DMF was gently stirred with NEt_3 (1.1 ml); if the solid did not dissolve within 4 to 5 min, or if the complex had crystallized out (recognized by its light yellow-orange color in comparison to the orange-red of bilirubin), dropwise addition of Et_2NH (up to 0.35—0.5 ml) caused the solid to dissolve. The orange-red solution (after filtration was transferred to a dish, and concentrated in a vacuum desiccator (10 torr) for 36 h. The orange-red crystals were carefully separated, washed with DMF and ether, and dried; yield, 370—385 mg. The product thus obtained was essentially devoid of the pink-fluorescing impurity, as shown by t.l.c. on cellulose; however, it still contained impurities extractable with 5% aqueous sodium bicarbonate.

Bilirubin (twice-recrystallized, 350 mg) was dissolved in chloroform (550 ml) at 55 °C by stirring in the dark. The solution was cooled to room temperature, successively washed with freshly prepared, 5% aqueous sodium bicarbonate solution (4 X 100 ml) and water (5 X 150 ml), and dried with

16

sodium sulfate (75 g) with vigorous stirring while slowly
adding the desiccant (30 to 45 min). The suspension was
filtered through a layer of microcrystalline cellulose mixed
with acid-washed carbon, and the filtrate concentrated
at 40 °C to incipient crystallization ($\sim$110 ml). Addition
of ether (30–40 ml) and cooling overnight gave crop I,
200 mg; crop II, 55–60 mg (total 255–260 mg). It showed
an ε_{mM} of 60.8.

This bilirubin (250 mg) was dissolved in chloroform
(350 ml) at 50 °C, cooled to 30 °C, and introduced onto
a column of anhydrous sodium sulfate (135 g) protected
from light by wrappings of aluminum foil. Complete elution
and washing (250 ml of chloroform) required 12 h. The
effluent was filtered, and concentrated to incipient
crystallization ($\sim$100 ml); addition of diethyl ether and
cooling (18 h) gave an orange-red, microcrystalline product;
yield 155 mg. Dilution of the mother liquor with some
ether, and cooling, gave crop II, 70 mg; total 225 mg. The
product was devoid of the pink-fluorescing impurity
(t.l.c.), and showed $\varepsilon_{mM}^{CHCl_3}$ 61.1. It contained 0.8% of
chloroform by chlorine analysis.

     g.   Sodium Borohydride Procedure: Alternative
         Method for Removal of the Fluorescent Impurity
         from Commercial Bilirubin

(A. J. Fatiadi)

This procedure depends on (<u>a</u>) the dissolution of
bilirubin in a methanol solution of sodium borohydride,
(<u>b</u>) the relatively low pH of the solution after the
addition of water, and (<u>c</u>) selective extraction by
chloroform of the impurity responsible for the fluorescence.
Recovery of the bilirubin from the alkaline solution by
acidification is followed, without intermediate recrystal-
lizations, by the bicarbonate extraction and sodium sulfate

treatments. The best results in this procedure were obtained with 0.1 to 0.2 g of bilirubin, although the procedure is described for 1.0 g of material.

Sodium borohydride (1.0 g) was quickly added to a stirred suspension of bilirubin (1.0 g) in methanol (250 ml, precooled to ~10 °C). The solids dissolved, sometimes with effervesence, in 30 to 45 sec, to give an orange-red solution. The stirred solution was diluted with water (250 ml; the solution showed pH ~8.0), and filtered (Millipore). The filtrate was extracted with chloroform (10 X 100 ml). The aqueous methanol layer was transferred to a large separatory funnel containing 1.2 liters of fresh chloroform, and acidified (to Congo Red paper) with 2.5 $\underline{M}$ hydrochloric acid (12 to 15 ml). By shaking, the precipitated bilirubin was dissolved in the chloroform layer. After a second extraction of the aqueous layer with chloroform (300 ml), the chloroform extracts were combined, washed with 5% aqueous sodium bicarbonate (6 X 250 ml), water (4 X 500 ml), and then dried by stirring vigorously with small additions (30—45 min) of sodium sulfate (total 100 g). The suspension was then filtered through a precoated layer of microcrystalline cellulose (10.0 g) mixed with acid-washed decolorizing carbon (2.0 g). This filtrate was concentrated under diminished pressure to incipient crystallization (~100 ml), saturated with ethyl ether (40—50 ml), and placed in a refrigerator for 18 h, to give a crop I (500 mg). Further concentration and cooling gave crop II (150 mg), total yield, 650 mg. Further purification by extraction with sodium bicarbonate and passage through a column of sodium sulfate was performed as described in the preceding procedure. T.l.c. analysis showed only a trace of the pink-fluorescing impurity (366-nm irradiation).

h.   Evidence for Presence of Two Additional
     Fluorescent Substances

(A. J. Fatiadi)

The presence of additional fluorescing substances was observed on examining the chloroform extracts obtained from the purification of 2.0 g of commercial bilirubin by the borohydride method. Bilirubin (2.0 g) in methanol (300 ml) at 10 °C was treated with sodium borohydride (1.2 g); the deep-orange solution was diluted with water (300 ml), filtered, and extracted with chloroform (6 X 150 ml). The combined extract was washed with water (3 X 150 ml), dried by stirring with 140 g of sodium sulfate for 30 min, filtered, and concentrated to about 10 ml; addition of a few ml of ether and slight cooling gave crude bilirubin (∿8 mg) which was filtered off. The yellowish-orange filtrate was concentrated to ∿5 ml and examined by t.l.c.

Spotting of 10- to 50 µl of solution on a cellulose plate and developing with methanol revealed three fluorescent spots: one at R_f ∿0.95 (strong orange-red fluorescence under 366-nm irradiation), one at R_f 0.90 (pink fluorescence), and one at the origin (a strong, light-blue fluorescence). The one at the origin can be readily moved by developing with chloroform. (The light blue fluorescent spot moves with the solvent front, and leaves a yellow, non-fluorescent residue at the origin.)

i.   Isomeric Forms of Bilirubin

The effort to estimate the proportion of the fluorescent impurity in the SRM from comparisons of the spectrophotometric properties of the SRM with those of SRM freed from this impurity, assumed that the bilirubin IXα from natural sources remains bilirubin IXα on purification. However, in the latter part of June 1971 we learned from Dr. A. F. McDonagh (University of California, San Francisco) that he and F. Assisi

were preparing to publish the discovery of the IIIα and XIIIα isomers in commercial preparations of bilirubin. They used t.l.c. on silica gel with 1:99 (v/v) acetic acid-chloroform. They found the highest proportions of these unexpected isomers in bilirubin from Pfanstiehl, the supplier of the bilirubin that is certified as the SRM. As the spectrophotometric properties McDonagh and Assisi had found for each bilirubin isomer were somewhat different, further work toward estimating the fluorescent impurity in the SRM would have to take into account the proportions of the isomers and their properties.

6. Standard Solutions of Bilirubin for Clinical Analysis

(B. Johnson and B. West)

In NBS Technical Note 547, we described our efforts to that time to find an optimal procedure by which to prepare standard solutions of the SRM bilirubin. Results which related mainly to the use of pooled human serum as the matrix for the standard solutions, were discussed at a meeting of the Expert Panel on Bilirubin of the International Federation of Clinical Chemists in Aarhus, Denmark, in August, 1970.

Some suggestions were raised at that meeting. These included (a) examination of the use of 5% solution (w/v) of human serum albumin (in place of the pooled serum), (b) use of tris(hydroxymethyl)aminomethane (Tris) buffer at pH 8.5 for dissolving the protein, (c) combination of the bilirubin concentrate and the protein solution at that pH, and (d) lowering the ratio of bilirubin to albumin to 1:10 (which would limit the maximum obtainable concentration of bilirubin). These suggestions became the basis for our further work.

20

a. Stability of Solutions of Bilirubin in
Albumin—Tris Buffer at pH 8.5; Comparison
with Two Enriching Solutions

A 5% solution of human serum albumin in pH 8.5 Tris
buffer was divided into several portions, and each was
enriched with SRM bilirubin that was freshly dissolved in
either 0.1 $\underline{M}$ potassium cyanide-formamide (KCN-F) [8] or
in methyl sulfoxide (Me_2SO) [9]. Albumin from only one
commercial lot was used, and enrichments were made on
different days. As usual in our work, the volume of
bilirubin solution added was limited to 2% of the final
volume of the enriched albumin solution. Each enriched
solution was tested (with albumin used as the blank) at
the time of enrichment and during the next several days
by direct spectrophotometry and by diazo coupling [10].
The results showed that the solutions prepared with
bilirubin in 0.1 $\underline{M}$ KCN-F were not stable. However, the
solutions enriched with bilirubin in Me_2SO were much more
stable. The latter solutions also exhibited higher
absorbances in both tests (see figs. 1 and 2). Solutions
with KCN-F were not studied further.

b. Effect of Storage Conditions on the
Stability of Bilirubin--Albumin Solutions

A fresh solution of albumin in pH 8.5 Tris buffer was
enriched using bilirubin—Me_2SO and divided as small
aliquots into three groups for storage under different
conditions. One group of aliquots was stored at +2 °C
in a refrigerator, a second at -20 °C in a freezer, being
cooled from room temperature to -20 °C in the freezer,
and the third group was quick-frozen by immersion in
Dry Ice—acetone, and immediately transferred to the freezer
at -20 °C.
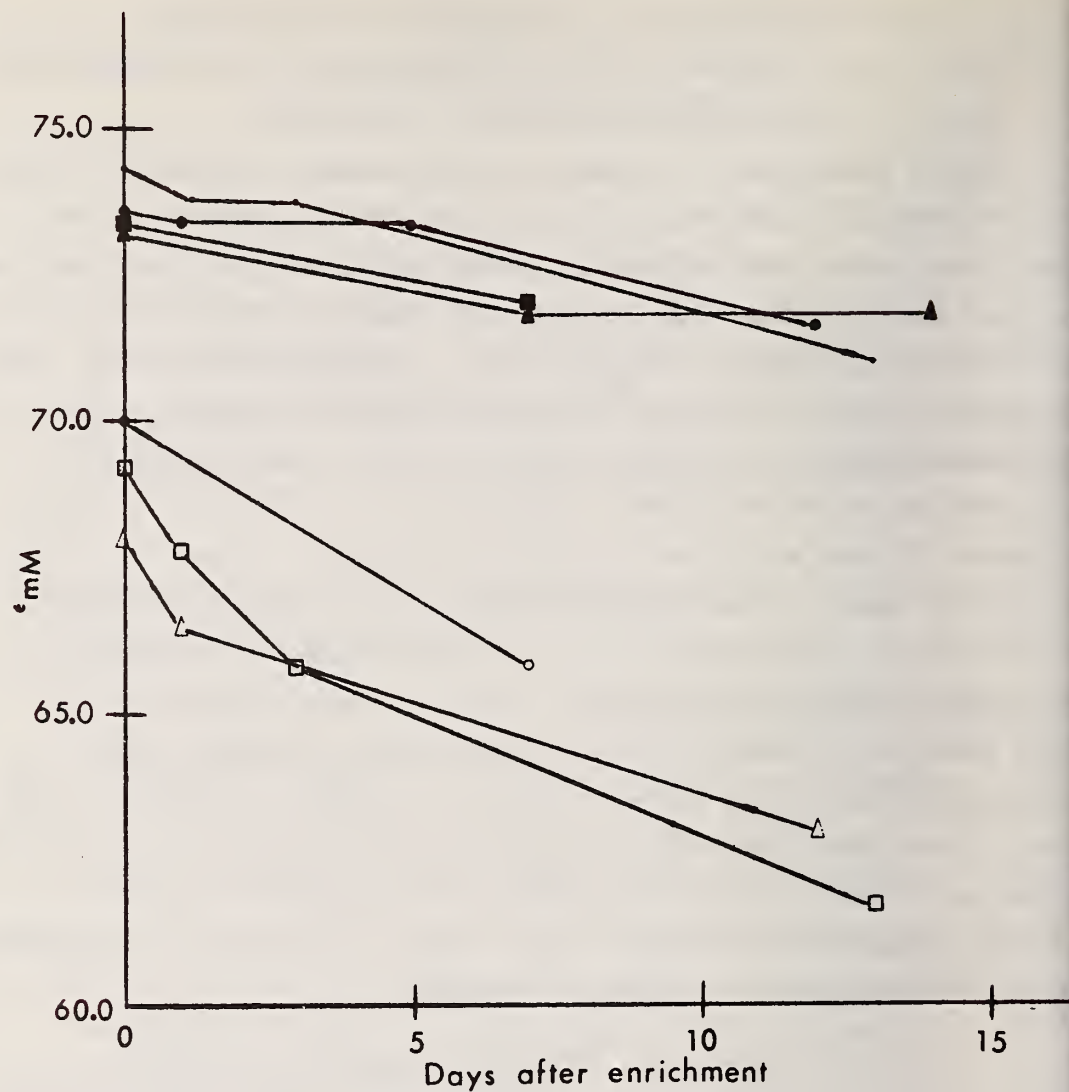
Figure 1.   Stability of several bilirubin—human albumin
            solutions prepared with (a) methyl sulfoxide
            (closed symbols) or (b) KCN-F (open symbols),
            as determined by the Jendrassik—Grof diazo test.

22
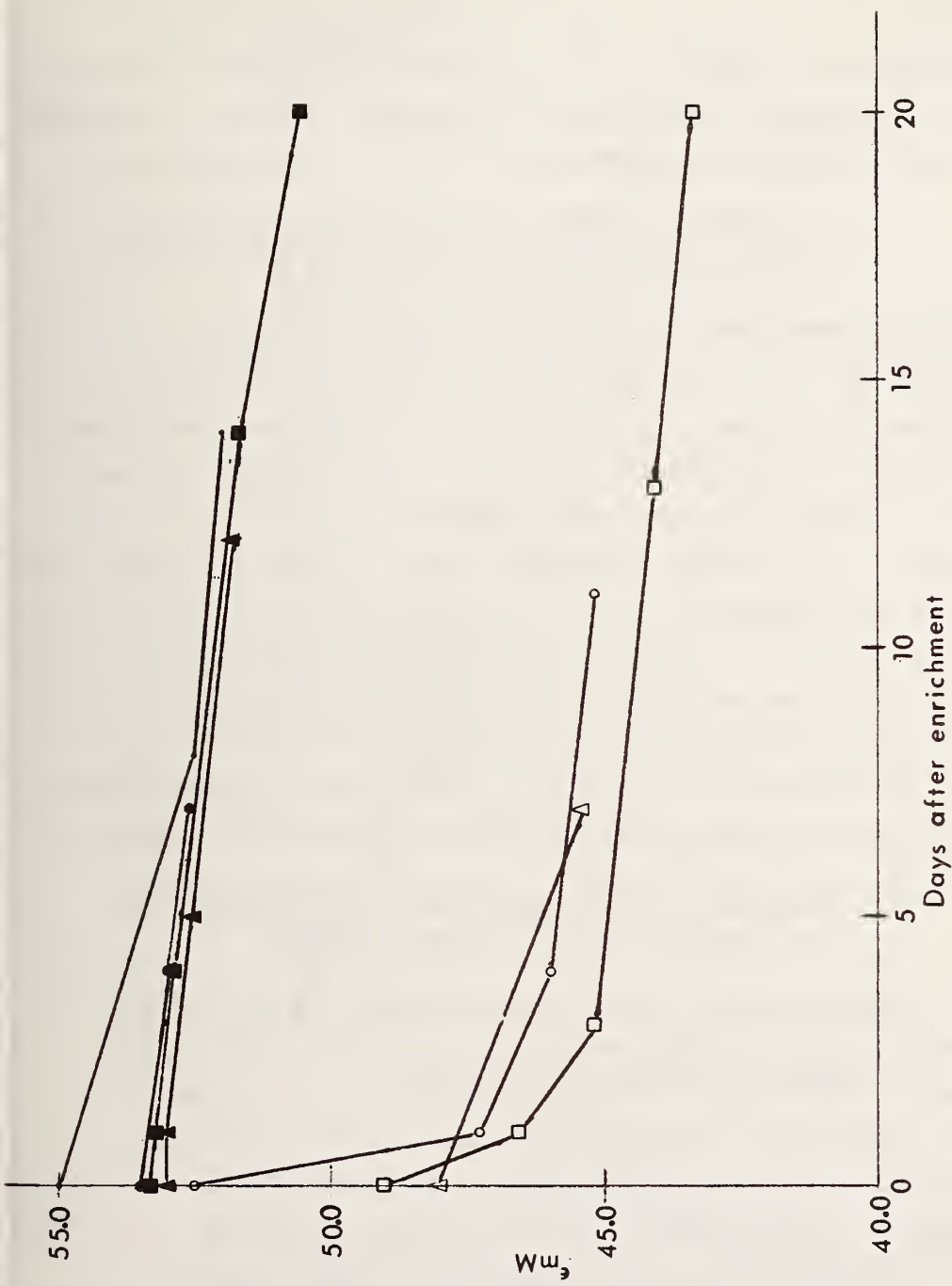
Figure 2. Stability of several bilirubin-human albumin solutions prepared with (a) methyl sulfoxide (closed symbols) or (b) KNC-F (open symbols), as determined by direct spectrophotometry.

23

The results from direct spectrophotometry and diazo
testing indicated that samples stored under all three
conditions underwent very similar rates of change over
the 7-day period of the study. Absorbances by direct
spectrophotometry declined by 1.5% and those from the diazo
test by 1.1% during the 7-day test period. Newly prepared
samples were stored thereafter at -20 °C in a freezer.

c. Calibration Curves for Bilirubin—Albumin
Solutions; Enrichment at pH 8.5

Bilirubin—albumin solutions having different
concentrations of bilirubin were prepared as follows: A
5% solution of albumin in pH 8.5 Tris buffer was enriched
with bilirubin in Me_2SO to a level of 6.4 mg/100 ml, and
aliquots of this solution were diluted 1:2, 1:4, and 1:8
with additional albumin solution. The solutions were tested
on the day of preparation only. Results by direct spectro-
photometry and diazo coupling gave fairly consistent values
for the millimolar absorptivity (ε_{mM}); by direct spectro-
photometry the average was 52.7; by the diazo test, 72.4.
The data, illustrated in fig. 3, showed that the standard
solutions would provide satisfactory calibration curves.

d. Bilirubin—Methyl Sulfoxide Enrichments
of Albumin in pH 7.4 Tris Buffer

The properties of solutions prepared with albumin
dissolved in pH 7.4 Tris buffer and enriched with bili-
rubin—Me_2SO were next examined. Analysis of stored
aliquots showed that samples prepared at this pH are
stable for at least six days (the duration of the
experiment). Enrichment at this pH had no effect on
the "azobilirubin" millimolar absorptivity. However, it
was found that, if samples are diluted with pH 7.4 Tris
buffer for direct spectrophotometric measurement, the ε_{mM}
is only 48.7; but, if pH 8.5 buffer is used as the diluent,

24
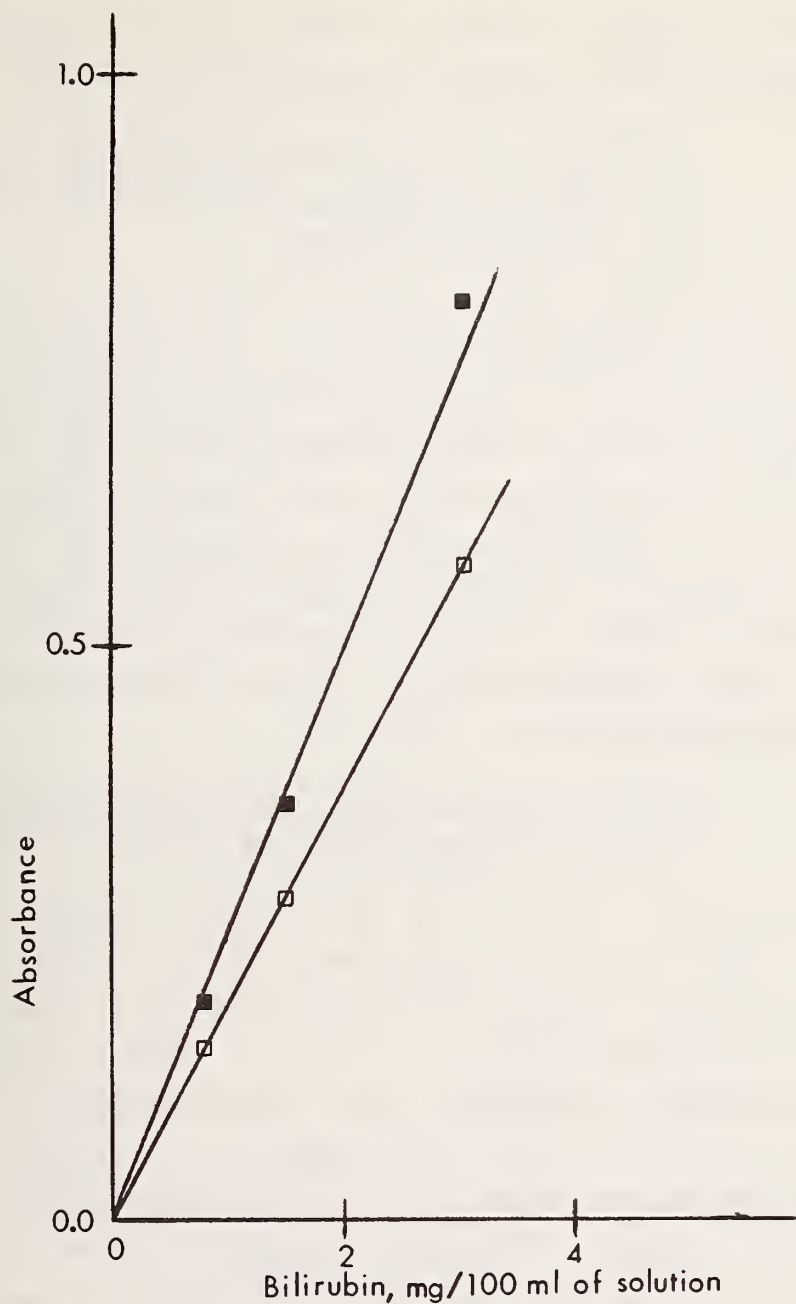
Figure 3.   Calibration curves for solutions of human albumin
            at pH 8.5 enriched with bilirubin in methyl
            sulfoxide. The upper curve is for the
            Jendrassik–Grof diazo test, and the lower is
            for direct spectrophotometry.

25

the $\varepsilon_{\text{m}\underline{\text{M}}}$ has the more "normal" value of 52.0. Presumably,
the binding on the albumin is more uniform at the higher pH.

e. Stability of Enriched Albumin Solutions (1) Enriched at pH 8.5, and (2) Neutralized to pH 7.4 Before Storage

Two enrichments of 5% solutions of human serum albumin
in pH 8.5 Tris buffer were made with bilirubin—Me$_2$SO.
Before storing at -20 °C, one of the solutions was
neutralized to pH 7.4 with $\underline{\text{M}}$ hydrochloric acid; the other
was kept at pH 8.5. For both, pH 8.5 Tris buffer was used
as the diluent before direct spectrophotometry. Over the
21-day test-period, the neutralized solution was found to
be essentially stable while the unneutralized solution
showed a total decrease in $\varepsilon_{\text{m}\underline{\text{M}}}$ (by both direct spectrophoto-
metry and diazo test) of 3.3%.

f. Calibration Curves for Bilirubin—Albumin Solution; Enrichment at pH 7.4

A 5% albumin solution at pH 7.4 was enriched to a
level of 17.50 mg of bilirubin per 100 ml of enriched
albumin solution. Aliquots of this solution were diluted
with additional 5% albumin (pH 7.4) to make a series of
bilirubin—albumin standards. The solutions were stored
overnight at -20 °C before the analyses were performed.
Dilutions for direct spectrophotometry employed pH 8.5
Tris buffer. Plots of absorbance versus concentration
of standard for both direct spectrophotometric and diazo
tests gave straight lines (see fig. 4). The average $\varepsilon_{\text{m}\underline{\text{M}}}$
as determined by direct spectrophotometry was 52.2 and by
diazo test was 71.3.

g. Comparison of Commercial, Human Albumin Preparations

It was of interest to determine whether lyophilized
human albumin (fraction V) from different commercial

26
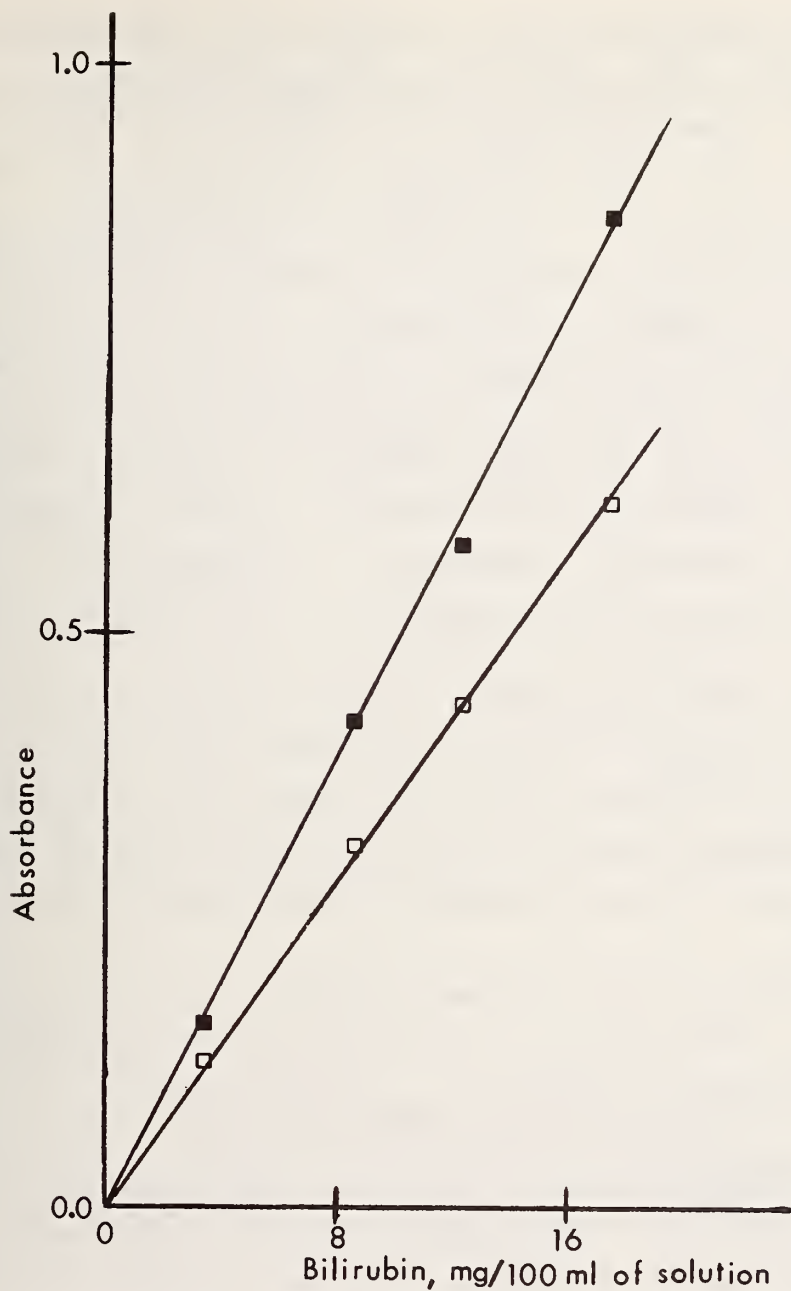
Figure 4. Calibration curves for solutions prepared by
enriching human albumin at pH 7.4 with bilirubin
in methyl sulfoxide. The upper curve is for
the Jendrassik—Grof diazo test, and the lower
is for direct spectrophotometry.

sources, or different lots from the same source, might significantly affect the values of ε_{mM} obtained in our tests. Six commercial samples and one sample supplied to us by Dr. A. Hainline (Center for Disease Control) were utilized.

There were variations in the physical appearance of the different albumin preparations. The more discolored, yellow materials were flaky in texture; one sample was gray-white and granular; and other samples were pale to off-white in color and fine textured. The more colored solids gave straw-colored solutions, and the lighter samples gave almost colorless solutions. A spectrum from 700 to 250 nm of the most yellow sample as a 5% solution showed an absorbance of 0.15 at 450 nm and of 0.30 at 410 nm. At a 1:5 dilution (the concentration used in direct spectrometry and diazo coupling of bilirubin), these absorbances fell to 0.03 and 0.06, respectively. The spectrum profile was a slowly increasing value of absorbance from 700 to 300 nm and then a more rapid increase in absorbance at shorter wavelength.

With some of these albumin samples, only one solution was tested. The results are summarized in table 2. Direct spectrophotometry showed the ε_{mM} varying from 52.0 to 55.6, with the λ_{max} ranging between 460 to 465 nm. Diazo testing showed ε_{mM} varying from 72.6 to 76.8, with the λ_{max} ranging from 595 to 600 nm. Although not directly apparent from these data, the use of lighter colored albumins led to somewhat higher values of ε_{mM} and, for direct spectrophotometry, to the shifting of the λ_{max} to longer wavelengths.

28

Table 2. Values of $\varepsilon_{m\underline{M}}$ from testing of bilirubin—methyl sulfoxide enrichments of different commercial samples of human serum albumin

Albumin lot number	Bilirubin (mg/100 ml)	Direct spectro-photometry		Diazo testing	
		$\varepsilon_{m\underline{M}}$	λmax	$\varepsilon_{m\underline{M}}$	λmax
27	16	53.6	460	74.6	595
132	4	55.6	465	75.1	598
133	4	52.0	460	73.4	598—600
401X	16	53.6	463	75.8	597
1849	16	52.5	463	72.6	597
none	4	54.3	465	75.4	597
CDC	3	54.7	464	76.8	599

B. Cholesterol

(R. Schaffer, A. Cohen, and R. F. Brady, Jr.)

In Technical Notes 457, 507, and 547, the analysis and shelf-life stability of cholesterol were reported. With the present data, it can be concluded that, after 3 years, the bulk SRM cholesterol continues to maintain its stability.

1. Thin-layer Chromatography of Cholesterol Samples

(A. Cohen)

Unidimensional chromatography on activated Silica Gel G plates with 1:1 (v/v) ethyl acetate—heptane was described in Technical Note 457 [11].

29

a. Differentiation Between Impurities and Artifacts by Two-dimensional, Thin-layer Chromatography

The impurities in the cholesterol, and artifacts formed during chromatography in the above were differentiated by two-dimensional t.l.c. Cholesterol (160 μg) was spotted from a solution in chloroform, on an activated plate (250-μm thick), developed for a distance of 10 cm in a saturated tank, dried rapidly with gaseous nitrogen, and then redeveloped in the second direction with the same developer. After spraying with 5:1 (v/v) methanol—sulfuric acid and charring, three spots were visible at R_f 0.61 (first), 0.56 (second); at R_f 0.44, 0.06; and at R_f 0.44, 0.48. The first of these spots is an impurity. The second is an artifact; it conforms in shape and has the R_f value found after unidimensional development of an equal amount of cholesterol with the same developer. In the direction from this artifact to the intense spot at R_f 0.44, 0.48, no other spots were detected, even under 366-nm light. Other impurities on the two-dimensional chromatogram were detected by their faint fluorescence, observed with both reflected and transmitted 366-nm light, along a line from the origin (R_f 0.0) through the intense spot at R_f 0.44, 0.48. The results are summarized in table 3.

Thus, the spots detected on unidimensional chromatography, other than those of the cholesterol and the artifact, the spot at the origin, are considered to be those of impurities.

Table 3.   Two-dimensional t.l.c. of freezer-stored
             cholesterol on Silica Gel G

R_f value[a]		Light used for detection[b]	Remarks	Identification[c]
1	2			
0.61	0.56	vis	weak	U
0.44	0.06	vis	faint	A
0.44	0.48	vis	strong	C
0.29	0.32	uv	faint	U
0.24	0.26	uv	faint	U
0.12	0.15	uv	trace[d]	U
0.00	0.00	uv	faint	U

[a]Key: 1, first direction; 2, second direction.
[b]vis, visible; uv, visible under 366-nm light.
[c]Key: A, artifact; C, cholesterol; U, unknown.
[d]One spot, or two.

b.   On Aluminum Oxide G—Silver Nitrate

T.l.c. was also performed on chromatoplates (250-μm
thick) of 33:10 (w/w) aluminum oxide G—silver nitrate [13].
Freshly prepared plates were allowed to dry overnight,
activated for 2 h at 120 °C, and stored over phosphorus
pentaoxide. Chloroform was used as the developer. The
bulk, frozen cholesterol was examined after spotting 20- to
160-μg quantities from a chloroform solution. The chromato-
plate was developed 10 cm, dried, sprayed with methanol—
sulfuric acid, and charred for 15 min at 120 °C. Elongated,
yellow spots appeared whose length was related to the
amount of cholesterol applied; these spots fluoresced
yellow. On further spraying with methanolic sulfuric acid
and charring for an additional 15 min, the spots became
brown and fluoresced pink. All spots tailed to the origin,
where a faint spot was present; a separate experiment,

31

employing two-dimensional chromatography, suggested that
this was an artifact. There were no indications of other
components.

    2.   Post-issuance Studies

        a.   Thin-layer Chromatography

(A. Cohen)

    Samples of the bulk supply of cholesterol that had
been stored in a freezer for 35 months showed no significant
evidence of change, but samples in loosely capped, brown
bottles that had been exposed to air at room temperature
in the laboratory for 30 months were found to be less pure.
Cholesterol that had been packaged under nitrogen, sealed,
and stored at room temperature for 22 months appeared
almost comparable to the freezer-stored cholesterol.

        b.   Gas—liquid Chromatography

(A. Cohen)

    G.l.c. was performed with a Varian Aerograph Series
2100 gas chromatograph equipped with a flame-ionization
detector and a glass column (180 X 0.2 cm i.d.) packed with
Gas Chrom Q (60—80 mesh, pretreated with 3% of OV-17) as
the stationary phase, heated to 240 °C, with the injector
and detector set at 260 °C. Chloroform solutions of
cholesterol ($\sim$10 µg/µl) were injected.

    In earlier studies of the SRM, g.l.c. was performed
with a Glowall Corporation Chromolab 320 Gas Chromatograph
fitted with a column (180 X 0.2 cm i.d.) with the same
phases, but operated at 240 °C. Solutions of cholesterol in
ethyl acetate were injected [12].

    With the Varian chromatograph, two peaks were detected
having the same retention times, 0.5 and 1.8, relative
to cholesterol as revealed with the Glowall instrument.
This pattern was found with samples of the bulk cholesterol

32

kept in a freezer for 35 months, with a packaged sample
stored at 20 °C for 22 months, and with an unpackaged sample
exposed to air and light for 32 months. There were no
peaks at 1.2 and 1.6, as had been observed with the
Glowall instrument, but a distortion in the tailing of
the cholesterol peak was noticeable at 1.2.

c. Ultraviolet Spectrophotometry

(A. Cohen)

Cholesterol exposed to air for 36 months at 20 °C in
a loosely capped, brown bottle was examined as a 1% solution
in dichloromethane; it showed very substantial absorption,
with a shoulder at ~245 nm that could be observed only after
dilution. In contrast, a 1.0% solution of SRM cholesterol
stored for 39 months in the freezer showed a simple
spectrum similar to that originally observed.

d. Differential Scanning Calorimetry (DSC)

(R. F. Brady, Jr.)

Five separate samples of the freezer-stored SRM
cholesterol (39 months after the first issuance of the
Standard Reference Material) were tested. The melting-
point curves were processed in the usual way [14]. The
results are given in table 4, where ΔH_f is the molar heat
of fusion, T° is the melting point in kelvins, and ΔT is
the melting-point depression. The purity values obtained
are like those previously obtained with the SRM.

Table 4. Reproducibility of the purity of SRM cholesterol as determined by differential scanning calorimetry

ΔH_f (cal/mol)	ΔH_f (J/mol)	T^0 (K)	ΔT (K)	Purity (%)
7940	33,200	419.67	0.07	99.85
7870	32,900	419.76	0.10	99.78
8020	33,600	419.77	0.10	99.77
7750	32,400	419.56	0.03	99.93
8190	34,300	419.76	0.13	99.69

e.   Phase Solubility Analysis

(R. F. Brady, Jr.)

Solubility analyses of three samples of freezer-stored cholesterol were made 34 months after the first issuance of the standard Reference Material. For a description of the technique used, see reference [15]. The cholesterol was found to be 99.80, 99.68, and 99.64 weight-percent pure in the three determinations.

f.   Volatile Matter in SRM Cholesterol by Mass Spectrometry

A Bell and Howell-Consolidated Electrodynamics Corporation (CEC), double-focusing, mass spectrometer Model 21-103 was used. The ionization potential was 70 eV, and the ion-source temperature was 250 °C. A sample (223 mg) of SRM cholesterol that had been stored in the freezer for ∿43 months was examined (by J. M. Ives of Section 310.05) for impurities by heating from ∿80 to 170 °C, and analyzing for any material that vaporized. Only water in a

very small proportion was released from the cholesterol under
these conditions. No fragments indicative of decomposition
of the cholesterol were found.

C.   Cortisol

<p style="text-align:right">(R. F. Brady, Jr.)</p>

In Technical Note 547, some of the early survey work
done on the cortisol standard was described. Since that
report, cortisol suitable for use as a Standard Reference
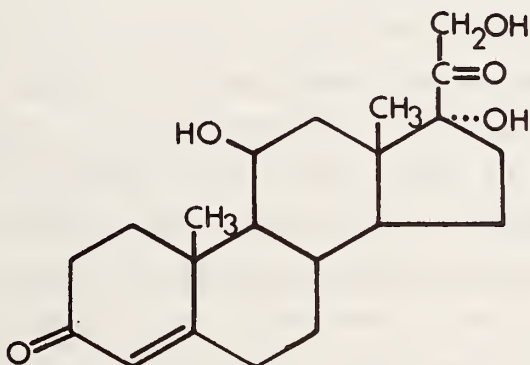
Material has been obtained, and work leading to its certifi-
cation was begun.

1.   Testing for Homogeneity

Nine samples were withdrawn from the bulk material
according to a statistical plan, and analyzed by use of
a commercial carbon—hydrogen analyzer. The standard
deviation for nine analyses was 0.16% for carbon, and 0.13%
for hydrogen; these values are well within the 0.3% limit
to the precision of the analytical method, and thus, the
standard material is homogeneous in respect to carbon and
hydrogen.

The specific optical rotation at five wavelengths, and the ultraviolet spectrum were measured in 95% ethanol for each of the nine samples. The data are given in table 5. The uniformity of these data is indicative of the homogeneity of the sample.

2. Elemental Composition

The analysis for carbon and hydrogen was made on four large samples of the standard material, and showed (±2 SD) carbon, 69.49 ±0.21%, and hydrogen, 8.39 ±0.09%. The theoretical values are: carbon, 69.58%, and hydrogen, 8.34%.

3. Behavior on Melting

The compound melted at 219.0 to 220.5 °C (corr.) when heated in an open capillary tube at 0.5 °C/min, and produced a pale-yellow melt that did not resolidify. When sealed under vacuum in a capillary tube, the material melted at 220.5 to 221.5 °C without yellowing, and did not resolidify. Thermogravimetric analysis of the sample heated in a dry nitrogen atmosphere at 2 °C/min showed, at about 221 °C (uncorrected), the initiation of the loss of a large proportion of sample weight; in air, however, the loss of weight began at 204 °C. Attempted use of differential scanning calorimetry showed that thermal curves obtained under nitrogen were not independent of heating rate or total heating time.

4. Spectrometric Data

The infrared and nuclear magnetic resonance spectra of the sample were identical with those reported earlier [16, 17]; neither technique gave evidence of impurity.

Table 5.   Specific optical rotations and millimolar
            extinction coefficients of SRM cortisol

	Wavelength (nm)					
Sample No.	589	578	546	436	365	242
	Specific optical rotation (degrees)					$\varepsilon_{m\underline{M}}$ at λmax
1	+167.1	+175.2	+205.7	+390.5	+576.2	16.2
2	+167.0	+175.4	+204.7	+388.4	+573.7	16.1
3	+167.1	+175.1	+205.4	+389.1	+575.6	16.1
4	+166.9	+175.3	+204.1	+389.7	+574.8	16.1
5	+167.1	+174.9	+204.9	+388.8	+575.9	16.2
6	+167.0	+175.4	+205.1	+380.0	+576.1	16.1
7	+167.2	+175.2	+205.5	+389.3	+575.7	16.1
8	+167.2	+175.2	+205.5	+389.3	+575.7	16.1
9	+166.9	+174.8	+204.4	+388.7	+575.1	16.1
Average	+167.0	+175.1	+205.2	+389.3	+575.3	16.1
1 SD	0.1	0.2	0.5	0.7	0.6	0.020

5. <u>Loss on Drying</u>

Three portions of cortisol were heated at 110 °C and 0.1
torr for three 24-hour periods. An average of 0.083% of the
sample weight was lost in the first 24-hour period, and
virtually no weight loss occurred on further heating. The
data are given in table 6.

Table 6.   Volatile matter in SRM cortisol

Weight (g)	Sample No.		
	1	2	3
Initial	2.814093	2.719680	2.747191
After 24 h	2.812008	2.717195	2.744883
% loss	0.074	0.091	0.084
After 48 h	2.811995	2.717186	2.744868
% loss	0.075	0.092	0.084
After 72 h	2.811986	2.717180	2.744856
% loss	0.075	0.092	0.085

---

6.   Insoluble Matter

Solutions of cortisol (1.8—2.0 g) in prefiltered
methyl sulfoxide (9—20 ml) were filtered through tared
Millipore filters having a pore size of 1.5 μm. Reweighing
of the filters after drying indicated a gain in weight
corresponding to 0.001% of the weight of cortisol used.
The stability of the filter membrane and the solvent blank
were determined by passing prefiltered methyl sulfoxide
through preweighed membranes. The average gain in weight
of the three membranes was 1 μg.

D.   D-Glucose   (Clinical)

(B. Coxon)

D-Glucose has now been issued as Standard Reference
Material 917, but some work in addition to that described
in previous reports [19, 20] has been done towards (a) the
certification of this material, and (b) confirmation of the
anomerization noted during previous analyses [20] by
differential scanning calorimetry.

Gas—liquid chromatography of trimethylsilyl derivatives
prepared from the crystalline Standard Reference Material
indicated [20] that it contained 99.5 percent of α-D-glucose

and 0.5 percent of the β anomer. However, similar analyses of material that had been partially or completely melted under the conditions of determination of purity by differential scanning calorimetry demonstrated that anomerization of the initial preponderance of α anomer occurs rapidly to give a mixture containing a slight excess of β anomer [20]. In the present work, the proportions of α- and β-D-glucose in the syrups prepared by melting them have been determined by p.m.r. spectroscopy at 90 MHz and correlated with the results from polarimetry of solutions of these materials at five different wavelengths. These techniques were performed by using solutions of the D-glucose materials in either methyl sulfoxide or methyl sulfoxide-d_6, for which our work showed that neither α-D-glucose nor β-D-glucose (nor mixtures of the two anomers) undergoes significant anomerization during the first 1.5 h following dissolution.

1. Analysis of D-Glucose by P.m.r. Spectroscopy at 90 MHz

The most useful signals for the analysis of α- and β-D-glucose are those of the anomeric hydroxyl proton, which are well separated at lowest field. The Bruker n.m.r. spectrometer used in this work was sufficiently sensitive to allow proportions of the minor anomer as small as 0.9 percent to be detected in a single scan of the spectrum. However, signal averaging (16 scans) was employed to improve the precision of the data. For solutions in pure, dry methyl sulfoxide-d_6, the signals of the anomeric hydroxyl protons occur as doublets, because of spin coupling with the proton (H-1) bound to C-1 of the sugar. Under these conditions, anomerization and rapid chemical exchange of the hydroxyl protons are suppressed by strong hydrogen-bonding with the solvent. The partial spectra obtained from solutions of crystalline α- and β-D-glucose are shown

in figure 5 (a and b). The spectra of solutions of melted
α- and β-$\underline{D}$-glucose in figure 5 (c and d) are practically
identical, and provide direct confirmation that, not only
do both α-$\underline{D}$-glucose and β-$\underline{D}$-glucose anomerize during
melting, but they do so to give equilibrated mixtures of
anomers. The quantitative proportions of anomers, obtained
by digital integration of all of these spectra within 4096
channels of a Fabritek instrument computer, are shown in
table 7. The proportions of anomers thus determined for
the melts are similar to those determined earlier [20] by
g.l.c. analysis of small quantities of α-$\underline{D}$-glucose that
had been melted completely in a differential scanning
calorimeter.

Table 7. Compositions of α- and β-$\underline{D}$-glucose and their
supercooled melts, determined by digital
integration of time-averaged, p.m.r. spectra[a]

	Content of	
$\underline{D}$-Glucose	α-$\underline{D}$-glucose	β-$\underline{D}$-glucose
	(%)	(%)
Crystalline α-	99.14	0.86[b]
Crystalline β-	0.97	99.03
Melted α-	47.4	52.6
Melted β-	47.9	52.1

[a]16 scans were accumulated for a solution of each material
in methyl sulfoxide-d_6. [b]Standard deviation of five
individual determinations, 0.11.

2. Polarimetric Analysis of Crystalline and Melted $\underline{D}$-Glucose Samples

Further confirmation of anomerization during melting,
to give an equilibrium mixture, was obtained from

Figure 5. P.m.r. spectra (16 scans) of the anomeric-hydroxyl proton region of solutions of D-glucose in methyl sulfoxide-d_6 at 90 MHz; (a) crystalline α-D-glucose, (b) crystalline β-D-glucose, (c) melted α-D-glucose, and (d) melted β-D-glucose.

polarimetric studies of solutions, in methyl sulfoxide, of crystalline and melted $\underline{D}$-glucose samples; it was found that the optical rotation values of melted α- and β-$\underline{D}$-glucose samples are essentially the same for each of five different wavelengths, and also that these values are intermediate between the values for the unmelted crystalline anomers.

The mole fractions of anomers determined by p.m.r. spectroscopy for the crystalline α- and β-$\underline{D}$-glucose were then substituted in simultaneous equations connecting mole fractions and optical rotations, and these were used to calculate the optical rotations of theoretically pure α- and β-$\underline{D}$-glucose for each of five wavelenghts. These rotations were then used in other simultaneous, mole-fraction equations connecting the specific rotations of the melted samples, so as to permit calculation of the mole-fractions of anomers present in these samples. For five different wavelengths, the mean mole-fraction of α anomer calculated to be present in the melted α-$\underline{D}$-glucose was 0.4324, and for the melted β-$\underline{D}$-glucose, this mole-fraction was 0.4320. These fractions are quite close to, but do not agree precisely with, the contents of α anomer in the melts determined directly by p.m.r. (see table 7), probably because of the assumption (used in the polarimetric calculations) that the melts contain only the α and β anomers. In fact, a small amount of caramelization of the sugars occurred during melting, and this may slightly modify the optical rotations observed for the melts.

In summary, the anomerization of crystalline α- and β-$\underline{D}$-glucose on melting, to give the same equilibrated mixture of these anomers, has been confirmed by both p.m.r. spectroscopy and polarimetry.

Full experimental details of the characterization of the $\underline{D}$-glucose SRM, and relevant numerical data and discussion, are given in a paper that has been published [21].

E. Synthesis of DL-4-Hydroxy-3-methoxymandelic Acid (DL-Vanillylmandelic Acid, VMA)

(A. J. Fatiadi)

In connection with a program on clinical standards being conducted at the NBS, there was need for a simple, practical method for the large-scale preparation of pure VMA in high yield.

A literature survey revealed that VMA had been prepared in 19 [22] to 45 [23] percent yield by a multiple-step procedure from vanillin via the cyanohydrin intermediate.

1. An Improved Procedure for Synthesis of VMA

An alternative route for the synthesis of VMA (4) is based on the procedure of Goodman and coworkers [24], who made use of the reaction previously employed in the synthesis of vanillin [25], in which guaiacol (1) is condensed with glyoxylic acid (2).
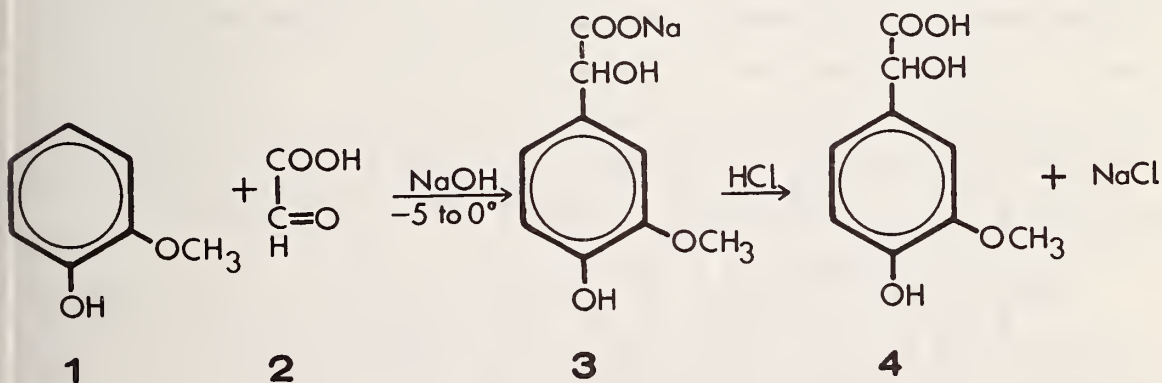
Our procedure for preparation of 4 entails slow addition of an ice-cold, aqueous solution of 2 to an ice-cold alkaline solution of 1, with efficient mechanical stirring. This one-step condensation provides VMA in 68–75 percent yield. The procedure has been described in a paper ready for publication.

F. D-Mannitol (Clinical)

(B. Coxon)

This work was directed towards certification of a
D-mannitol SRM for use in the clinical analysis of
triglycerides present in sera. The D-mannitol serves
the function of providing, on periodate oxidation, a
standard source of formaldehyde with which can be compared
the formaldehyde produced by similar oxidation of the
glycerol liberated on saponification of the triglycerides.

A preliminary screening of four commercial samples
of D-mannitol by thin-layer chromatography, paper
chromatography, and optical rotation studies was described
in Technical Note 547 [26].

1. Synthesis of D-Mannitol for Use as a Reference Sample Within These Laboratories

Most commercial D-mannitol is prepared either by
electrolytic or catalytic reduction of alkaline solutions
of D-glucose, and, before purification, the D-mannitol may
contain [27] as much as 1.5—2.5% of sorbitol (D-glucitol).
Thus, we anticipated that, on a small scale, the reduction
of D-mannose with sodium borohydride might more quickly
provide a pure sample of D-mannitol than many recrystal-
lizations of commercial D-mannitol.

To a cooled solution of recrystallized D-mannose (30 g)
in water (90 ml) was added dropwise, with magnetic stirring,
a solution of sodium borohydride (10 g) in water (150 ml)
during 0.5 h. The mixture was stirred for a further 0.75 h;
a negative test for reducing sugar was then obtained with
boiling Fehling solution. Washed, regenerated, Amberlite
IR-120 (H^+) ion-exchange resin was then gradually added and,
when the effervescence had ceased, ∿200 ml of this resin was
added, followed by Norit. The suspension was stirred for
1 h at room temperature, and was then filtered through Celite
that had been thoroughly prewashed with water. The filtrate

44

was concentrated to a syrupy suspension that was evaporated under diminished pressure with three 400-ml portions of methanol, and then twice at atmospheric pressure with 500 ml of methanol, to remove boric acid as methyl borate; a negative flame-test for methyl borate was then obtained. The preparation, evaporated to dryness, was dissolved in hot water (50 ml), hot ethanol (500 ml) was added, and the product was allowed to crystallize at room temperature and then in a refrigerator. The crystals of D-mannitol were filtered off, washed twice with ethanol, and dried under vacuum at 60 °C; yield 26.5 g (87%), mp 166.9—167.9 °C. Three recrystallizations from 5:1 (v/v) ethanol—water (600 ml) gave D-mannitol* as globular clusters of colorless needles, wt. 19.2 g, mp 167.1—168.0 °C.

D-Glucitol was prepared in a similar way by reduction of SRM D-glucose. After two recrystallizations from ethanol, and drying under vacuum at 55 °C, the D-glucitol showed premelting at 87—89 °C, and melted at 95—96 °C.

2. Determination of Purity by Phase Solubility Analysis

(E. Hohertz)

Pyrex test-tubes (2 X 15 cm) were cleaned by successive washing with soap solution, tap water, distilled water, and acetone. After being dried, the tubes were constricted near the open end by drawing them out in a natural gas—oxygen flame. Samples of D-mannitol (0.1, 0.3, 0.7, 1, 2, 3, and 4 g) were placed in each of 7 tubes, together with 1:1 (v/v) methanol—water (10 ml). The tubes were sealed in a gas—oxygen flame, shaken in a bath at 26 °C for six days, and then opened and centrifuged.

---

*Referred to hereinafter as reference D-mannitol.

Aliquots (2.0 ml) were removed from each supernatant solution and allowed to evaporate at room temperature in tared, aluminum pans. The pans and contents were then dried under vacuum at 50 °C. From the weights of these contents and the initial sample-weights, a phase-solubility diagram was plotted. The results for purity are compared in table 8 with those derived by differential scanning calorimetry.

3. Determination of Purity by Differential Scanning Calorimetry [28]

Typically, each sample of D-mannitol (∿0.5 mg) was weighed in an aluminum pan which was then hermetically sealed and heated in a Perkin-Elmer Model DSC-1B differential scanning calorimeter from 380 to 436 K at 80 K/min, and then from 436 to 442 K (beyond melting) at 0.625 K/min, by use of the maximum sensitivity (range 1) of the calorimeter. The areas of the thermograms of the D-mannitol studied for certification as a Standard Reference Material required a correction of only 1.5–3% in order to linearize the plot of temperature versus the reciprocal of the fraction melted. The purity values obtained by DSC are compared in table 8 with those measured by phase solubility analysis. The data shown in table 9 indicate that the purity values obtained for D-mannitol by DSC are not significantly dependent on the temperature (T) at which slow scanning is begun.

4. Optical Rotation of Samples of D-Mannitol

D-Mannitol (0.4 g) was dissolved in freshly prepared, aqueous, 5% ammonium molybdate tetrahydrate solution (20.0 ml); the solution was diluted with 0.5 M sulfuric acid [29] to 25.0 ml at 20 °C.

46

Table 8.   Purity values[a] obtained for D-mannitol by
           differential scanning calorimetry (DSC) and
           phase solubility analysis (PSA)

   (B. Coxon, R. F. Brady, Jr., J. H. Thomas, and E.
    Hohertz)

Manufacturer	Purity, mole % (DSC)	Purity, weight-% (PSA)[b]
1	99.60	99.73
2	99.74	99.62
3	99.60, 99.63	99.67
4	99.68	99.78
4, experimental sample[c]	see table 12	99.78
4, test sample	99.91, 99.91	
4, SRM test sample	99.91	
4, SRM sample [d]	99.94	99.74, 99.85 [1:1 (v/v) ethanol—water]][b]
[e]	99.94	
[f]	99.92	
Reference		
D-mannitol [c]	99.84	99.95

[a]These values should not be considered to be equally
reliable. [b]The crystals were extracted with 1:1 (v/v)
methanol—water, unless otherwise stated. [c]These samples
of D-mannitol were prepared by reduction of D-mannose with
sodium borohydride. [d]From top of supply. [e]From middle of
supply. [f]From bottom of supply.

Table 9. Purity values obtained for D-mannitol by
          differential scanning calorimetry, with slow
          scanning begun at various temperatures (T)

(R. F. Brady, Jr., and E. C. Douglass)

Manufacturer	Purity, mole %	T (K)
4	99.88, 98.80	431
	99.91, 99.91	433
	99.75, 98.34	434
	99.98	435
	99.65, 98.90	436
	99.94	438
4, experimental sample[a]	99.80	429
	99.75	430
	99.86	433
	99.91	436
Reference sample of D-mannitol[a]	99.92	431
	99.89	435
	99.92	436
	99.98	438

[a]These samples of D-mannitol were prepared by reduction of
D-mannose with sodium borohydride.

Measurements were made on freshly prepared solutions
at 20 °C by use of a thermostated, 1-dm cell in a
Perkin-Elmer Model 141 polarimeter. The values of specific
rotation obtained from various samples are shown in
table 10.

Table 10. Specific rotations, at various wavelengths, of
solutions of various samples of $\underline{\underline{D}}$-mannitol in
acidified ammonium molybdate solution

$$[\alpha]_\lambda^{20} \text{ (degrees) of}$$

Wavelength (λ), in nm	SRM test sample	1[a]	SRM samples 2[b]	3[c]
589 (Na-D)[d]	+138.7, 138.9	+139.0	+139.4	+139.0 (139.0)[e]
578	145.2, 145.2	145.8	145.2	146.0
546	166.3, 165.0	166.3	166.8	166.1
436	297.1, 296.9	298.6	299.0	298.2
365	___f	___f		
Concn.	1.60, 1.62	1.60	1.60	1.61 (1.60)

	Recrystallized SRM sample[g]	Mother liquors from the recrystallization
489 (Na-D)[d]	+139.4	+139.0
578	145.8	145.3
546	165.5	166.2
436	298.0	297.8
365	___f	___f
Concn.	1.60	1.60

---

[a] From top of supply.
[b] From middle of supply.
[c] From bottom of supply.
[d] Literature value [30], $[\alpha]_D^{20}$ +139.2 ±0.3° (c 1.60).
[e] The value in parentheses is the mean of 10 readings measured
by the use of a Rudolph, high-precision, manual polarimeter.
[f] Values were not obtained at this wavelength, because of
strong absorption by the solution.
[g] Recrystallized from 1:1 (v/v) methanol-water.

Small values of optical rotation were obtained for solutions of various samples of D̠-mannitol in water (see table 11).

Table 11. Specific rotations, at various wavelengths, of solutions of samples of D̠-mannitol in water

Wavelength (λ), in nm	$[\alpha]_\lambda^{20}$ (degrees)		
	of SRM test sample	of SRM sample 2[a]	Literature value
389 (Na-D)	-0.4[b]	-0.3[c]	-0.2 to -0.5
578	-0.5	-0.2	—
546	-0.5	-0.5	—
436	-1.3	-1.1	—
365	-3.1	-2.8	—
Concn.	10.03	16.03	7 to 16.6

---

[a] D̠-Mannitol from middle of supply.
[b] Unchanged after 16 h at 20 °C.
[c] Mean of 10 readings.

5. Thin-layer Chromatography (T.L.C.) of D̠-Mannitol

a. Partition T.L.C. on Layers of Silica Gel G

T.l.c. of the test sample, SRM test sample, and SRM samples of manufacturer 4, and of the reference D̠-mannitol (0.5—2.0 µl of 10% solution in water) was conducted on either 20 X 20 cm layers, or on 20 X 40 cm layers of Silica Gel G developed in the 40-cm direction for 13—16 h. The following solvent mixtures were used for development: ethyl acetate—isopropyl alcohol—water (1:1:1, 1:2:1, 1:2:2, 1:3:1, 1:2:3, and 6:2:1 v/v), butyl alcohol equilibrated with 1:1:1 (v/v), 3 M̲ ammonium hydroxide—methanol—water and 1:1 (v/v), methanol-saturated sodium tetraborate

solution. Although slight separation of D-glucitol from D-mannitol was achieved with some of these solvent mixtures, mixtures of these alditols containing 1% or 5% of D-glucitol were not usually reproducibly resolved. However, these experiments served to confirm the absence of significant amounts of organic impurities having mobilities different from those of D-mannitol and D-glucitol. Spraying of the t.l.c. plates with 5% sulfuric acid and then heating at 120 °C revealed D-mannitol as a brown spot, and D-glucitol as a black spot.

b. Adsorption T.L.C. on Layers of Calcium Sulfate

Initially, 0.2–2 μl of aqueous, 10% solutions of the SRM D-mannitol samples, of D-glucitol, and of D-mannitol containing 1, 5, and 50% of D-glucitol were applied to layers (5 X 20 cm) of calcium sulfate (for t.l.c.) that had been activated at 110 °C for 0.5 h. Activation at higher temperatures or for longer times caused cracking or peeling of the layer from the glass plate. On the other hand, the use of non-activated layers gave poor results, with much streaking.

The plates were developed with 1:1:3 (v/v) acetic acid–benzene–methanol. However, development of spots of D-mannitol that had been applied from aqueous solution resulted in the appearance of a separate, artifactual spot that remained at the origin. This spot was virtually absent when solutions of the D-mannitol in methanol were applied to the plate and then developed. The appearance of an artifactual spot after spotting of aqueous solutions is apparently due to rehydration of the calcium sulfate layer ($CaSO_4 \cdot 0.5\ H_2O \rightarrow CaSO_4 \cdot 2H_2O$), and subsequent partition of the D-mannitol between the water remaining at the origin (not removed by drying at 40 °C under vacuum) and D-mannitol adsorbed on the layer and developed by the solvent. The use of methanol as solvent is not particularly convenient,

51

because of the low solubility of D-mannitol in it (<0.2%); this necessitates multiple spotting in order to deposit sufficient material on the plate.

By the use of this technique, proportions of D-glucitol in the D-mannitol at the 50% level could be detected, but not at the 1% and 5% levels. Evidently, this phenomenon is due to the fact that a slower-moving, large amount of D-glucitol causes a slightly faster-moving, large amount of D-mannitol to move farther along the plate than if none, or only a small amount, of the D-glucitol is present, i.e., saturation of the adsorption capacity of the layer.

6. Paper Chromatography of D-Mannitol

This technique was performed as described previously [26], except that larger volumes (20 µl) of aqueous 10% solution were also applied to the paper. No impurities were detected in the samples of SRM D-mannitol by this technique, although D-glucitol was not well resolved under these conditions.

7. Ultraviolet Spectrophotometry of D-Mannitol

(B. A. Johnson and B. Coxon).

Samples (2 g) of the SRM D-mannitol from the top, middle, and bottom of the supply, of the recrystallized SRM D-mannitol, and of the solid obtained by evaporation of the mother liquor of this recrystallization (see later) were each made up to a volume of 10.0 ml with distilled water.

The ultraviolet spectra of the solutions of SRM samples showed only a low background absorbance (0.01—0.02) in the region of 380—240 nm. However, between 219 and 200 nm, the absorbance increased very rapidly to a strong, end absorption. The spectrum of the recrystallized SRM D-mannitol was similar, both as to shape and as to absorbance values. The spectrum of the solution of mother-liquor material showed in many regions more absorbance than

52

could be accounted for by the assumed enrichment-factor of 5 (see later). For example, at 290 nm, the mother liquors showed an absorbance of 0.1, whereas the SRM and recrystallized SRM samples displayed an absorbance of only ∿0.01. It seems probable that the mother liquors might have gained some absorbing material from the methanol or filter paper used in the recrystallization. In any case, no impurities were detected in the SRM D-mannitol by this method.

8. Infrared Spectroscopy of D-Mannitol

(B. Coxon, B. A. Johnson, and E. Hohertz)

The D-mannitol samples were examined as pellets prepared by pressing with potassium chloride. The samples of manufacturers 1–4, the SRM samples, the mother liquor material from recrystallization of the SRM sample, and the experimental sample from manufacturer 4 after recrystallization, all showed very similar infrared spectra, with a very strong, broad, hydroxyl absorption in the region of 3000–3500 cm^{-1}, C–H stretching absorptions of medium intensity in the 2900–3000-cm^{-1} region, and a relatively complex "fingerprint" region. In contrast, the spectra of the experimental sample from manufacturer 4 (prepared from D-mannose) and the SRM D-mannitol that had been recrystallized from aqueous methanol were similar to each other, but different from the aforementioned spectra, particularly in the C–H stretching and fingerprint regions. In further contrast, the spectra of the reference D-mannitol (long, fine needles), which had been recrystallized from aqueous ethanol, and of the sample of manufacturer 4 after recrystallization from the same solvent mixture, were similar to each other, but markedly different, with respect to band intensities and positions in the C–H and fingerprint regions, from all of the aforementioned spectra.

53

These results indicate that at least three polymorphic forms of D-mannitol have been encountered in the present work, but neither infrared spectroscopy nor proton or carbon-13 magnetic resonance spectroscopy indicated the presence of any impurities in the samples of SRM D-mannitol.

The infrared spectra of these samples were very similar to a published spectrum of D-mannitol [30].

9. Concentration, by Recrystallization, of Impurities in SRM D-Mannitol

The SRM D-mannitol (100.0 g of material taken in approximately equal amounts from the top, middle, and bottom of the supply) was dissolved in distilled water (400 ml) by warming to 34 °C, and methanol (400 ml) was added to the solution. After nucleation with D-mannitol, the solution was allowed to cool slowly from 34 °C to room temperature, and was kept at this temperature for several hours, and then in a refrigerator for three days. The large, crystalline clusters of colorless slivers obtained were washed with methanol, and dried at 60 °C in vacuo; yield 80.8 g. The mother liquor and washings were combined, and evaporated to a colorless solid (19.2 g). Thus, the maximum possible enrichment factor for impurities transferred from the SRM material to the mother liquor is approximately five.

The recrystallized material and the mother-liquor material were subjected to analysis by infrared spectroscopy, ultraviolet spectrophotometry, optical rotations in acidified ammonium molybdate solution, and gas-liquid chromatography of hexakis-O-(trifluoroacetyl) derivatives.

10. Loss of Weight of SRM D-Mannitol on Drying

    a. At 110 °C

(B. A. Johnson)

The samples of SRM D-mannitol (50 g, accurately weighed) in tared, aluminum-foil pans were dried in an oven at 110 °C and ~3 torr, and were periodically removed from the oven and allowed to cool for one hour over phosphorus pentoxide in a desiccator before being weighed. The weights lost are shown in table 12.

Table 12. Loss in weight of SRM D-mannitol on drying at 110 °C

| Drying time (h) | Loss in weight (mg) of samples | |
	Top sample (49.98700 g)	Bottom sample (49.51548 g)
17	4.14	3.53
37	6.11	5.90
57	7.26	7.14
80	10.88	11.01
Total weight lost, %	0.022	0.022

---

    b. At 70 °C

On drying at 70 °C for 24 h, 7-g samples from the top, middle, and bottom of the SRM supply lost 0.002, 0.001, and 0.002%, respectively.

The ash content of 25-g samples was found to be less than 0.001%, and therefore no determinations of trace inorganic constituents were made.

The material in the SRM D-mannitol (25-g samples) that was insoluble in water was found to be 0.0006, 0.0001, and 0.003% for the top, middle, and bottom samples, respectively.

Satisfactory carbon and hydrogen analyses were obtained for these samples.

G. β-Nicotinamide Adenine Dinucleotide (β-NAD) and its 1,4-Reduced Form (β-NADH)

(A. Cohen)

In Technical Note 547, some initial studies were described on the characterization of β-NAD, a compound relatively more stable than β-NADH. With that work as a background, we began a study of β-NADH, desired for issuance as a standard reference material.

1. Ultraviolet Spectral Data for Commercial Samples of β-NADH

The NRC values [31] of $\varepsilon_{m\underline{M}}$ for β-NADH at pH 10 are 14.4 at 259 nm and 6.2 at 340 nm; $\underline{A}_{260}/\underline{A}_{340}$ 2.32. It is generally accepted that low values of $\underline{A}_{260}/\underline{A}_{340}$ are indicative of purer β-NADH.

All samples were dried for at least 24 h under high vacuum at ∿20 °C. Lot 065003 was dried for 4 days. Two samples of lot 2549 were used; one was dried for 24 h and the other for 48 h. Lot 678-9 was obtained as a preweighed vial, and the weight given on the label ("5.0 mg") was used in the calculation of the millimolar absorptivity ($\varepsilon_{m\underline{M}}$) of the sample. The results are summarized in table 13.

a. β-NADH, Lot 50C-6420

Two samples of powdered β-NADH were obtained from the commercial source that supplied lot 678-9. The data sheet supplied with lot 50C-6420 indicated that the sample had a molecular weight of 791; this includes solvation by ∿3.5

56

Table 13. Ultraviolet spectral characteristics for different commercial samples of β-NADH in 0.1 M Tris buffer, pH ~10

$\varepsilon_{mM}{}^a$

Wave-length (nm)	NRC constants [31]	Lot 065003 Found	Given[b]	Lot 90176 Found	Given[b]	Lot V3837 Found	Given[b]	Lot 2549 Found[c]	Given[b]	Lot 678-9 Found	Given[b]
~235	---	6.86	---	6.66	---	6.64	---	6.70	6.73	6.81	---
259	14.4	14.7	14.6	14.6	14.5	14.3	14.1	14.7	14.8	14.8	---
260	---	14.7	---	14.6	---	14.4	---	14.8	14.8	14.8	---
~290	---	1.65	---	1.47	---	1.49	---	1.42	1.48	1.63	---
340	6.2	6.10	6.0	5.84	---	5.84	---	5.63	5.69	6.14	6.2

Absorbance ratio

	NRC constants [31]	Lot 065003 Found	Given[b]	Lot 90176 Found	Given[b]	Lot V3837 Found	Given[b]	Lot 2549 Found[c]	Given[b]	Lot 678-9 Found	Given[b]
A_{250}/A_{260}	0.82	0.79	0.80	0.80	0.80	0.79	0.82	0.80	0.80	0.79	0.81
A_{280}/A_{260}	0.23	0.26	0.25[d]	0.27	0.24	0.27	0.23	0.25	0.26	0.26	0.23
A_{260}/A_{340}	2.32	2.41	2.43	2.50	---	2.46	---	2.62	2.60	2.41	2.3

---

[a]Calculated by assuming a formula weight of 763.46.
[b]Manufacturer's data for this lot.
[c]No data supplied by the manufacturer.
[d]Calculated.

57

molecules of H_2O/molecule and 0.4 molecule of ethanol/
molecule. Although we examined the samples after they had
been dried under high vacuum, the molecular weight of 791
was used to calculate ε_{mM}. The sample weights used were 3.178
and 3.741 mg. The data obtained are given in table 14.

Table 14. Ultraviolet spectral data for β-NADH (lot no.
50C-6420) in 0.1 $\underline{M}$ Tris buffer, pH ∿10.4

Wavelength (nm)	Manufacturer's data	ε_{mM}	
235		6.86	6.96
259		14.9	15.1
260		15.0	15.1
290		1.57	1.62
340	6.20	6.00	6.10
		Absorbance ratio	
$\underline{A}_{260}/\underline{A}_{340}$	2.3	2.49	2.48
$\underline{A}_{250}/\underline{A}_{260}$	0.81	0.79	0.81
$\underline{A}_{280}/\underline{A}_{260}$	0.23	0.25	0.26

2. Paper Chromatography

From our previous chromatographic studies, the
fluorescent intensity of a solution of β-NADH in 0.1 $\underline{M}$ Tris
buffer (pH ∿10) kept at ∿4 °C for 38 days appeared to be only
slightly greater than that of an unbuffered NADH solution in
25% ethanol. All samples of NADH were therefore dissolved in
0.1 $\underline{M}$ Tris that contained 25% of ethanol. The ethanol facili-
tated evaporation (with nitrogen gas) of solvent from the

spots applied, and also permitted the preparation of more-compact spots.

Each sample (300–320 μg) was spotted on Whatman No. 1 filter paper, and developed with 2:1 (v/v) pyridine–water [32] by descending chromatography. The R_f values and manufacturer's lot numbers are listed in table 15.

Identifications of NADPH and AMP were based only on the similarity of their R_f values to those of reference samples used as standards. Lots 065003 and V3837 seemed to contain more NADPH (based on a higher fluorescence intensity with 366-nm light) than the other samples. Lot 678-9 did not appear to show any evidence of NADPH for the amount spotted, but it had an impurity at R_f 0.35. "Adenosine diphosphoribose" sodium salt (ADPR) at R_f 0.73 and α-NADH at R_f 0.71 could not be distinguished from β-NADH, possibly because of the amount of β-NADH spotted.

Table 15. Paper chromatography of commercial samples of β-NADH

R_f values of components of lot no.					Detection by fluorescence		Identifi-cation
065003	90176	V3837	2549	678-9	under 366-nm light	after KOH spray [33]	
0.68	0.67	0.66	0.66	0.66	blue		β-NADH
0.57	0.56	0.56	0.54	0.55	none	blue	β-NAD
0.47w	0.46w	0.46w	0.45w	---	blue		NADPH[b]
0.43	0.43	0.42	0.42	0.42	none	none	AMP[c]
---	---	---	---	0.35vvw	blue		unknown
0.0	0.0	0.0	0.0	0.0	pale whitish		unknown

_____

[a] Key: v, very; w, weak.
[b] Reduced nicotinamide adenine dinucleotide phosphate, sodium salt.
[c] 5'-Adenylic acid.

In the chromatographic analysis for impurities given in table 15, the inspection was performed after the chromatographic sheets had been allowed to dry. To assure valid observation of properties of these impurities (loss of fluorescence on drying of the paper might lead to an incorrect identification or characterization), the chromatography was repeated, and the _wet_ sheets were examined and compared to the air-dried sheet. No differences were found

3. Loss of Weight on Drying, and Elemental Analysis

Approximately 200 mg of β-NADH from lot 50C-6420 was weighed accurately into an all-glass weighing container, and dried under high vacuum at $\sim$20 °C to constant weight. The loss amounted to 2.8%. The dried sample was analyzed for its elementary composition. The results are shown in table 16.

Table 16. Elemental analysis of a dried sample of β-NADH, lot 50C-6420

	Calculated				
For	C	H	N	P	Na
$C_{21}H_{29}N_7O_{14}P_2$	37.9	4.4	14.7	9.3	
$C_{21}H_{27}N_7Na_2O_{14}P_2$	35.6	3.8	13.8	8.7	6.5
2 H_2O	33.8	4.2	13.2	8.3	6.2
3 H_2O	33.0	4.4	12.8	8.1	6.0
3.5 H_2O	32.7	4.4	12.7	8.0	6.0
4 H_2O	32.3	4.5	12.6	7.9	5.9
Found	31.6	4.1	12.2	8.1	6.40; 6.22

60

4. Stability of β-NADH under Conditions for Enzymic Analysis

The reaction occurs as follows:

$$NADH_2 + CH_3CHO \rightleftharpoons NAD + CH_3CH_2OH$$
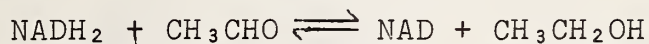
The method of Ciotti and Kaplan [34] was followed with use of the following reagents: 1:40 dilution of alcohol dehydrogenase, 0.5 $\underline{M}$ acetaldehyde, and 0.1 $\underline{M}$ potassium phosphate buffer (pH 7.5). The quantities used were increased by a factor of 33 for greater precision; hence, 100 ml of total solution was prepared. The change in absorbance at 340 nm was measured. The results are summarized in table 17.

Thus, the study of the enzymic analysis revealed possible sources of error, for example, the acetaldehyde should have been buffered before combination with the β-NADH. If a number of portions are to be taken from a container of dry β-NADH, this must be done in air having a low relative humidity, and the remaining β-NADH should be re-examined after additional drying under high vacuum. The slight difference of $\underline{A}_{260}/\underline{A}_{340}$ in the two samples of the 21-day, freezer-stored sample cannot be explained.

5. Inhibitor(s) in β-NADH

Samples of β-NADH may contain an inhibitor that affects kinetic studies with some enzymes. The inhibitor is stated to be produced when β-NADH is in contact with moisture.

a. Chromatographic Studies of β-NADH Treated with Moisture

i. Paper Chromatography

A sample of β-NADH from lot 065003 was exposed to moist air for 48 h; then it was dissolved in Tris buffer and chromatographed against unexposed β-NADH from lot 065003 and lot 678-9. The chromatographic characterization of these untreated commercial samples of β-NADH was reported in table 15.

61

Table 17. Stability[a] β-NADH (lot no. 065003) under conditions for an enzymic assay (after 4 days of drying at 20 °C under high vacuum); uv spectral results

	Mfr.'s data		I	II	III	IV	V	VI	VII
Buffer	Tris[b]	Tris	Tris	Phos-phate	Phos-phate	Phos-phate	Tris	Tris	Tris
Elapsed time (days)	0[c,d]	0[c,d]	21[c,d]	3[c,d]	10[c,d]	12[c] 14[d]	12[c] 16[d]	21[c,d]	22[c,d]
Wavelength (nm)		ε_{mM}[e] Found							
~235	---	6.86	6.78, 6.79	6.67	6.50, 6.46	6.60	6.69	6.74	6.88
260	14.6	14.7	14.7, 14.8	14.5	14.4, 14.4	14.4	14.5	14.7	15.0
~290	---	1.65	1.66, 1.68	1.59	1.66, 1.71	1.64	1.63	1.64	1.72
340	6.0	6.10	6.07, 6.00	5.96	5.61, 5.57	5.76	5.79	5.91	6.01
					Absorbance ratios				
A_{250}/A_{260}	0.80	0.79	0.79, 0.79	0.79	0.79, 0.78	0.80	0.79	0.80	0.80
$\underline{A}_{280}/\underline{A}_{260}$	0.25[f]	0.26	0.27, 0.27	0.26	0.28, 0.29	0.27	0.27	0.26	0.27
$\underline{A}_{260}/\underline{A}_{340}$	2.43	2.41	2.42, 2.47	2.44	2.57, 2.58	2.50	2.50	2.49	2.50
Purity (%)[g]	97				86[h], 85				

62

[a]Key and comments: I. Duplicate samples of the dried material were reexamined after storage for an additional 21 days in a freezer. The samples appeared to be stable.

II. The required volume of acetaldehyde solution was premixed with some of the buffer. This solution was used to transfer the weighed β-NADH to the 100-ml volumetric flask. The spectral values were obtained within 20 min; the value of A_{260}/A_{340} indicated stability of the solution for at least 20 minutes.

III. First, the required amount of acetaldehyde solution was added to the volumetric flask, and then the weighed sample of β-NADH was transferred with the aid of the phosphate buffer. The high value of A_{260}/A_{340} and the low value at 340 nm may have resulted from uncontrolled contact with the unbuffered acetaldehyde, which contained (according to the manufacturer) 0.01% of acetic acid; β-NADH is known to be unstable under acidic conditions. From the 100 ml of solution, 9.97 ml (calibrated pipet) was withdrawn, and combined with 0.33 ml (calibrated pipet) of the diluted enzyme solution. (The 1:40 dilution of the enzyme was used to avoid oxidation of any NADPH that might be present.) There was a rapid decrease in absorbance at 340 nm, and the absorbance appeared to be stable after 0.5 h. The residual material in the solution was fluorescent under 366-nm light.

IV. Acetaldehyde was not present in experiment IV. The higher absorptivity at 340 nm and the lower A_{260}/A_{340} compared to III, suggests that the unbuffered acetaldehyde solution initially put into the flask in III was destructive to the β-NADH added.

Comparison of the results listed in columns II and IV suggested that the dried β-NADH was undergoing some change, possibly caused by uptake of moisture from the air (from opening the sample vial several times for sampling during

a 12-day period). The vial had been stored in the dark in a desiccator, but not redried between samplings.

Comparison of Tris with phosphate buffer (in columns IV and V) indicates that, in the time used for the measurement, the phosphate-buffered solution was not a source of decomposition of the NADH. In IV and V, the samples were weighed out on the same day, but dissolved several days later for spectral examination.

In method VI, the sample was taken from the vial and dissolved in the buffer on the same day, and, in comparison with IV and V, higher values at 340 nm and 260 nm were obtained.

VII. The main vial of NADH from which the previous samples had been taken was redried under high vacuum (over phosphorus pentaoxide); the sample then taken had higher spectral values at 340 nm and 260 nm than by method VI.

[b] 0.1 $\underline{M}$ Tris buffer, pH ~10.4.
[c] Day when sample was weighed.
[d] Day when sample was dissolved for analysis.
[e] Based on disodium salt of β-NADH·3H$_2$O; molecular weight, 763.64.
[f] Calculated.
[g] Enzymic analysis.
[h] Horse-liver alcohol dehydrogenase, 213 U/mg [35].

The chromatogram of the exposed β-NADH (chromatography in 2:1 (v/v) pyridine-water) was examined under 366-nm light, and showed a highly fluorescent, blue zone from the vicinity corresponding to the $\underline{R}_f$ of the β-NAD to that of NADPH. It also showed a weak fluorescent impurity having an $\underline{R}_f$ corresponding to that in untreated lot 678-9. Treatment of the chromatographic sheet with alcoholic potassium hydroxide showed a large increase in a spot having the $\underline{R}_f$ of β-NAD (β-NAD or a ternary nicotinamide type of compound). The use of potassium hydroxide as an indicating spray was mentioned in table 15.

A 33-day old solution of moisture-exposed NADH was further examined in other chromatographic systems, with ultraviolet light and the alcoholic potassium hydroxide spray.

ii.   PEI-Cellulose

By use of 0.2 $\underline{M}$ Tris buffer (pH 9.0), no resolution occurred, except for β-NAD.

iii.   PEI-Bakerflex

With 0.2 $\underline{M}$ Tris buffer (pH 9.0) as developer, only resolution of β-NAD resulted.

iv.   Cellulose, Eastman Chromagram

With 2:1 (v/v) pyridine—water as the developer, NADPH is not resolved, but exposed β-NADH, as well as a fresh solution of β-NADH, was resolved into two additional fluorescent spots (366-nm light).

v.   DEAE-Cellulose (Bakerflex)

With 0.13 $\underline{M}$ aqueous ammonium hydrogen carbonate, traces of a fluorescent impurity (366-nm light) and of a quenched impurity (254-nm light) emerged from both the exposed and fresh solutions of β-NADH. There appeared to be a substantial increase in β-NAD in the moisture-exposed material, indicating that this is not due alone to the paper chromatography, which required 24 h. There was no resolution of a major fluorescent spot such as has been attributed to the inhibitor. A fresh solution of lot 678-9 NADH that had been exposed for 19.5 h, examined in this system, behaved essentially like the older sample. However, the quenched spot (254 nm) was somewhat more evident and may be related to ADPR.

The best resolution seemed to be obtained by use of 2:1 (v/v) pyridine—water on Whatman No. 1 filter paper or t.l.c. on cellulose.

b. Rate of Formation of Inhibitor as Determined by Chromatography

β-NADH samples were exposed to moisture during 1, 2, 3, 4, and 5 h, and their interaction was quenched by dissolving the material in 0.1 $\underline{M}$ Tris buffer (pH ~10). These solutions were then compared with an untreated, fresh control.

With DEAE-cellulose (Bakerflex) and 0.13 $\underline{M}$ ammonium hydrogen carbonate solution as the developer, no apparent differences could be observed.

By t.l.c. on cellulose, or overnight paper chromatography, with 2:1 (v/v) pyridine—water, no apparent differences were observed.

c. Column Chromatography

With a column of DEAE-cellulose pretreated with 1 m$\underline{M}$ ammonium hydrogen carbonate, β-NADH exposed for 8 days to moisture was applied as a solution in, and developed with, the same solvent and then, with 10 m$\underline{M}$ ammonium hydrogen carbonate. Only four well-separated, nonfluorescent fractions that did not appear to absorb at 340 nm were eluted.

Chromatography of three of the separate eluted materials, on PEI-cellulose with 0.2 $\underline{M}$ Tris buffer (pH 9), with subsequent examination by uv light (254 and 366 nm) and alcoholic potassium hydroxide spray, indicated that one of the fractions might consist of α,β-NAD, another might be nicotinamide; the third was not identified. From their $\underline{R}_f$ values, these fractions were not ADPR, NADP, or nicotinamide mononucleotide (NMN).

With the alcoholic potassium hydroxide spray NMN fluoresces, further confirming the usefulness of the KOH spray for compounds of the ternary nicotinamide type, and as a replacement for the potassium cyanide spray that has been used to detect such compounds.

H. Purification of Synthetic Dyes for Use as Spectrophotometric Standards

The Analytical Coordination Chemistry Section is working to develop liquid filters for use in checking the photometric scales of spectrophotometers. We are cooperating by developing procedures for purifying organic dyes to their specifications.

(A. J. Fatiadi)

Samples of the purified, synthetic dyes were needed as the reference material for the spectrophotometric standards. Three commercial dyes were checked, namely, Neolan Black (Ciba), Cibalan Black (Ciba), and Alizarine Light Gray (Allied).

T.l.c. studies on non-activiated silica gel plates with 3:1 or 5:1 (v/v) isopropyl alcohol–acetone showed a considerable proportion of trailing spots caused by impurities. Solubility studies showed the presence of a considerable proportion of neutral, inorganic components. All three dyes were readily soluble in water, and moderately to difficultly soluble in ethanol, acetone, or ethyl acetate. It was difficult to develop a general procedure for purification of all three dyes; hence, a specially designed procedure was necessary for each individual dye.

1. Neolan Black

Crude Neolan Black (1 g) was extracted in a Soxhlet apparatus with ethyl acetate (200 ml) for 3 h, and the residue was recrystallized from hot water (250 ml). Concentration of the aqueous filtrate under vacuum was difficult, because of foaming, even after addition of an antifoaming agent such as octyl alcohol. Addition of alcohol or acetone to the filtrate gave an amorphous product containing inorganic salts. The filtrate was concentrated to incipient crystallization at 85–90 °C

67

(water bath) under a stream of nitrogen, and left to
crystallize at room temperature. Washing with water, and
drying in a vacuum desiccator, gave dark-blue, lustrous
crystals (yield 50 percent). The product was homogeneous
by t.l.c.

2. Cibalan Black

A sample of the crude dye (1 g) was extracted in a
Soxhlet apparatus with acetone (1 liter) for 15 h; the
extract was filtered, and the filtrate concentrated almost
to dryness, to give lustrous crystals; yield 550 mg. The
product was recrystallized from acetone (with concentration)
and submitted for spectrophotometric testing; it was found
satisfactory as to absorption, and stability in aqueous
solution.

3. Alizarine Light Gray 2BLWN

The Color Index [36] generic name and number of this
dye are Acid Black 48, C.I. 65005.
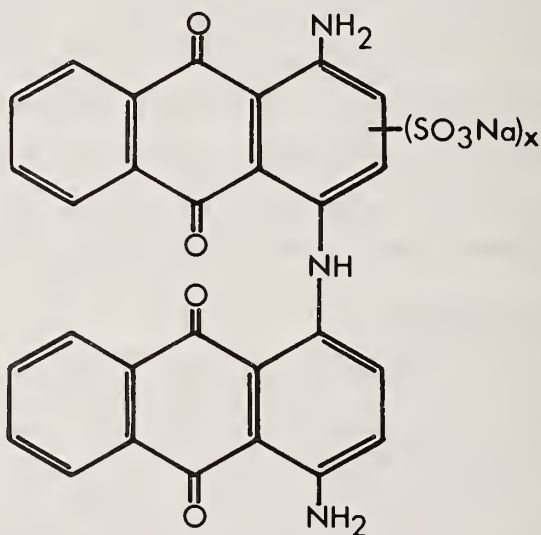
The crude dye had the following analysis: Na, 8.8; S, 3.9%.
Initially, purification was attempted by extraction with hot
water and concentration of the filtrate at 80 °C under a

stream of nitrogen to incipient crystallization. However, the lustrous product that crystallized out was difficult to filter off, requiring 10 to 12 h for a 1-g batch, and analyses for sulfur and sodium were not reproducible. The following procedure was therefore devised.

Commercial dye (8 g) was suspended in N,N-dimethyl-formamide (DMF) (70 ml) and stirred at 45 °C (water bath) for 20 min. The suspension was filtered, and the solids were washed with DMF (30 ml) to give a dark-blue solution. The filtrate and washings were combined, cooled to 20 °C, and treated, with stirring, with ethyl ether (25–30 ml); the precipitate (dark-blue microcrystals) was washed with ether, and dried in a vacuum desiccator at 78 °C/0.1 torr; yield 250 mg. The crystals had the following analysis. Found: Na, 4.4; S, 5.3%.

The filtrate was diluted with another portion of ether (60–80 ml), stirred at room temperature for 10 min, and precipitate 2 was obtained; yield 1.6–2.0 g, dried at 78 °C and 0.1 torr. Found: Na, 3.9; S, 5.5%. The filtrate was treated with more ether (60 ml), to give a third precipitate (0.2 g).

(A. Cohen)

A second portion of the crude dye (24 g) was purified by the procedure using DMF and ether. However, after the first precipitate had been collected, the mother liquor, without further addition of ethyl ether, was stored in a refrigerator, and a crop of crystalline dye deposited. Its spectrum in the visible range was judged superior to that of any other crop examined. The infrared spectrum of this crop of dye lacked a band at 6 μm that was found in all other crops; by elemental analysis, the preparation most closely corresponds in composition to a hydrated dye; see table 18.

Table 18.   Elemental analysis of the modified preparation
            of Alizarine Light Gray, dried at 20 °C and 1 torr

Percent calculated for $C_{28}H_{16}N_3NaO_7S$

|	C	H	N	Na	O	S
anhydrous	59.9	2.9	7.5	4.1	20.0	5.7
1 H_2O	58.0	3.1	7.3	4.0	22.1	5.5
1.5 H_2O	57.1	3.3	7.1	3.9	23.1	5.5
2 H_2O	56.3	3.4	7.0	3.9	24.1	5.4
2.5 H_2O	55.5	3.5	6.9	3.8	25.1	5.3
3 H_2O	54.6	3.6	6.8	3.7	26.0	5.2
Found	55.8	3.7	6.4	3.9	---	5.0

2. CARBOHYDRATE SYNTHESIS, STRUCTURE, AND CHARACTERIZATION:
    NOVEL RESEARCH MATERIALS AND MODEL COMPOUNDS

A. Enolization of Hexodiulose Acetals. Synthesis of
    Derivatives of D-Psicose by Reduction of a
    Hex-3-enulopyranose

                                    (R. F. Brady, Jr.)

    Previous papers from this laboratory have reported the
synthesis of isopropylidene acetals of D-erythro-pentulose
[37], D-threo-pentulose [37], D-fructose [38], and D-psicose
[39], and have demonstrated their utility in the synthesis
and purification of rare ketoses. This work describes an
attempt to synthesize similar acetals of D-tagatose
(D-lyxo-hexulose).

    1,2:4,5-Di-O-isopropylidene-β-D-erythro-2,3-hexodiulo-
2,6-pyranose 1, an intermediate previously used [39], was

70
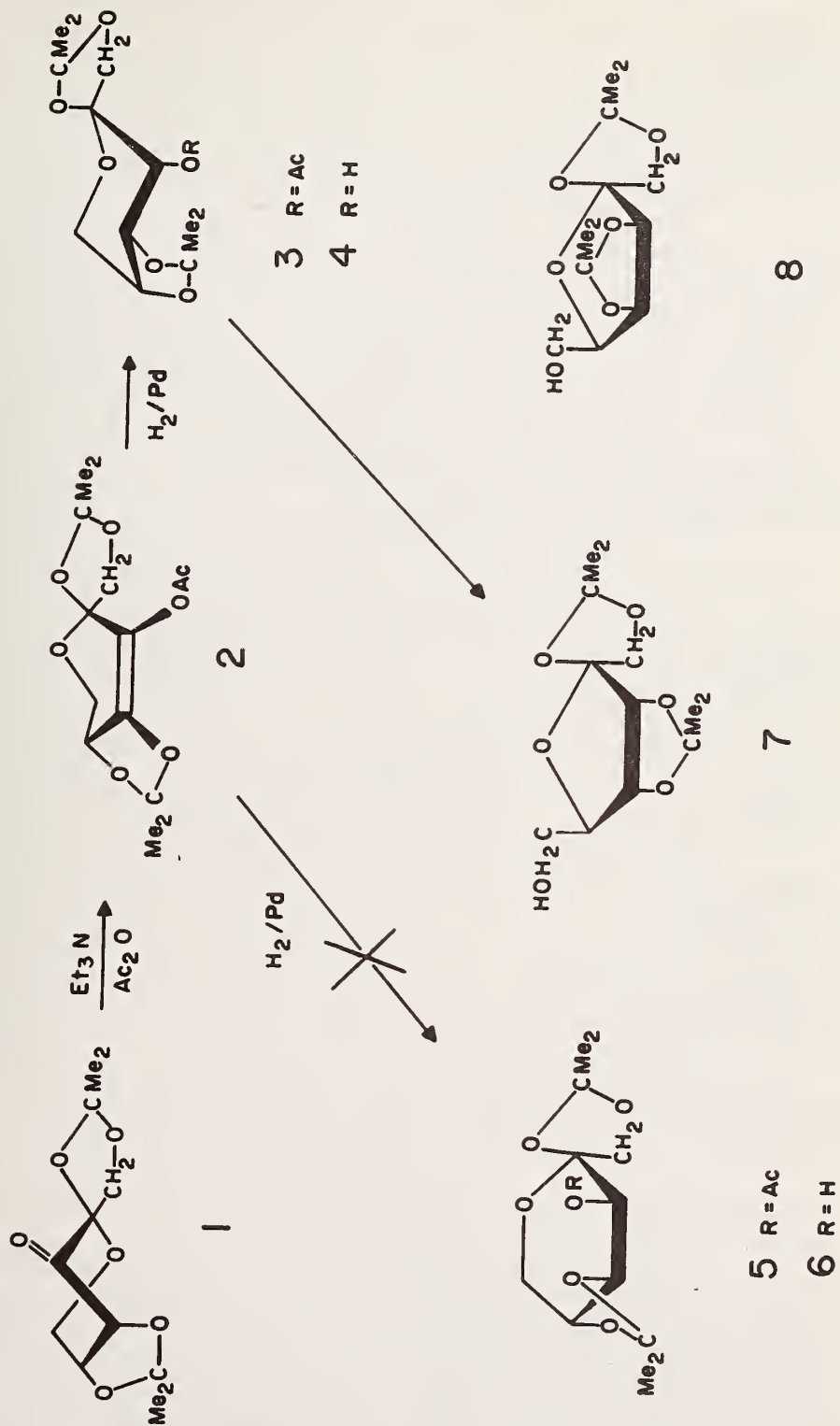
treated with an acetic anhydride—triethylamine reagent [40]
for 3 days at 50 °C, and gave 46% of a new, crystalline
compound whose i.r. spectrum showed bands at 1770 and
1746 cm^{-1}, indicative of a O-C=C-O-C=O system [40], and
whose elemental analysis was consistent with the formula
$C_{14}H_{20}O_7$. The n.m.r. spectrum showed the presence of two
isopropylidene acetal groups and one acetyl group; two
1-proton doublets for the protons on C-1, and a well-defined,
eleven-line, AMX pattern representing the protons on C-5
and C-6 were also readily identifiable. These data, and
the fact that diulose 1 has a proton on C-4 but not on C-2,
established that the product is 3-O-acetyl-1,2:4,5-
di-O-isopropylidene-β-D-glycero-hex-3-enulopyranose (2).

Attempts to prepare the enediol acetate 2 by treating
diulose 1 with acetic anhydride and pyridine (a) at room
temperature for 16 h, (b) under prolonged heating at 80 or
125 °C, or (c) under reflux for 1 h, were unsuccessful.
Treatment of diulose 1 in carbon tetrachloride solution
with an acetic anhydride—perchloric acid reagent [41] did
not give the desired product 2. Treatment of 1 in
tetrahydrofuran solution with an acetic anhydride—sulfuric
acid reagent [42], or slow distillation of the solvent from
a solution of diulose 1 in acetic anhydride containing
p-toluenesulfonic acid [43], also failed to produce enediol
acetate 2. Earlier attempts, using acidic conditions, to
prepare an enediol acetate from a precursor ketone having
acid-labile blocking groups were also unsuccessful [40].

On reduction of 2, cis-addition of hydrogen at C-3
and at C-4 might result in formation of the corresponding
derivative of D-tagatose or D-psicose, or both. Inspection
of a molecular model of compound 2 revealed that the
molecule could assume either an H_6^O conformation or that
sofa conformation [44] having the oxygen atom of the
pyranoid ring exoplanar to the plane of the carbon atoms

72

of this ring. Reduction of 2 at either side of either
conformation appeared possible.

One possible reduction product, namely, 3-O-acetyl-
1,2:4,5-di-O-isopropylidene-β-D-psicopyranose (3), would be
formed by cis-addition of hydrogen at that side of the plane,
defined by C-2—C-3—C-4—C-5, on which O-2 is located. By
analogy with the conformation [45] of 1,2:4,5-di-O-
isopropylidene-β-D-fructopyranose, compound 3 may be expected
to assume a conformation, intermediate between $1C(D)$ and
$H_0^2(D)$, in which that part of the molecule containing C-4,
C-5, and C-6 is slightly flattened from the ideal chair
conformation because of the presence of the 1,3-dioxolane
ring involving C-4 and C-5. The unacetylated compound 4
has been prepared and found to be stable [39].

The other possible reduction product, namely,
3-O-acetyl-1,2:4,5-di-O-isopropylidene-β-D-tagatopyranose (5),
would be formed by cis-addition of hydrogen to that side
of the plane on which C-1 is located. Diacetal 5 would
contain an isopropylidene group trans-fused to the equatorially
attached hydroxyl groups on C-4 and C-5. Although several
hexulopyranose derivatives containing a trans-fused
isopropylidene group have been reported [46], isopropylidene
groups trans-fused to six-membered rings generally cause
steric strain [47] in these rings, and are thus disfavored.
Neither diacetal 5 nor the unacetylated compound 6 has yet
been reported. In view of the steric strain in compound
5, similar strain in the transition state in the reduction
of 2 to 5 could also be anticipated.

Experimentally, it was found that reduction of 2 is
stereospecific, and that only diacetal 3 is formed.
Hydrogenation of compound 2 at atmospheric pressure in the
presence of 5% palladium-on-carbon catalyst gave a syrup that
was shown to be 3-O-acetyl-1,2:4,5-di-O-isopropylidene-β-
D-psicopyranose 3). Acetate 3 was deacetylated with methanolic
barium methoxide [48] to afford crystalline 1,2:4,5-di-O-

73

isopropylidene-β-D-psicopyranose (4). Diacetal 4 was converted into a furanose derivative by treatment with acetone containing 0.5% of sulfuric acid to effect rearrangement, giving a chromatographically homogeneous, levorotatory, crystalline compound, identified as 1,2:3,4-di-O-isopropylidene-D-psicofuranose (7). The corresponding derivative of D-tagatofuranose (8) is a strongly dextrorotatory, crystalline solid [49].

Reduction of the enediol acetate 2 with sodium borohydride in ethanolic solution is complete after 18 h, and affords diacetal 4 in high yield. This reaction evidently proceeds by reductive deacetylation of 2, with intermediate formation of the enolate anion; this ketonizes, and is protonated by the alcoholic solvent, forming diulose 1, which is subsequently reduced to diacetal 4. Compound 4 has also been obtained [39] by direct reduction of diulose 1 with borohydride. Although the reduction of 2 to 4 with sodium borohydride is much slower than similar borohydride reduction of an enediol acetate prepared by Horton and Just [40], the product obtained in both cases is the same as that

obtained when the diulose is reduced by borohydride directly. This suggests that the reduction proceeds through the diulose in each case.

As a synthetic route to isopropylidene acetals of D-psicose, the reduction of compound 2 is not preferred, because direct reduction of diulose 1 with borohydride gives diacetal 4 in 99% yield [39]. However, this synthesis of derivatives of D-psicose may prove valuable for the synthesis of acetals made with other aldehydes and ketones, in which the reduction of the diulose analogous to 1 would not be stereospecific because of bulkier alkylidene acetal groups.

B. **Application of Homonuclear, Internuclear, Double-resonance Techniques to Carbohydrates. Detection of Small Coupling-constants**

(B. Coxon)

1. Introduction

In previous reports [19, 20, 50], iterative analyses of the proton magnetic resonance spectra of a variety of derivatives of carbohydrates have been described. Such analyses are necessary for full characterization of these derivatives by n.m.r. spectroscopy, and also for the measurement of accurate coupling-constants and chemical shifts to be used in the assignment of conformations and conformational equilibria.

An important step in the iterative analysis of an experimental spectrum is the assignment of peaks in this spectrum to the theoretical transitions generated by the computer program for the analysis. From the point of view of the validity of the analysis, it is preferable if additional experimental evidence for these assignments is obtained from some type of multiple-resonance experiment.

2. Results and Discussion

In the present work, homonuclear, internuclear, double-resonance (indor) techniques [51] have been employed to confirm the spectral assignments for 6-deoxy-1,2:3,5-di-O-isopropylidene-α-$\underline{D}$-glucofuranose-$\underline{d}_{12}$($\underline{1}$-$\underline{d}_{12}$). The iterative analysis of this compound by magnetic-equivalence factoring has been described in a previous report [20]. The essential experimental requirement for an indor experiment in the frequency-sweep mode is that the measuring frequency ($\underline{f}_1$) be held constant at the peak of a resonance while the double-resonance frequency ($\underline{f}_2$) is swept through the frequencies of connected transitions. For example, in figure 6i is shown the complete, high-resolution, p.m.r. spectrum of

75

1-$\underset{\sim}{d}_{12}$, and in fig. 6a–6h are given the indor spectra obtained by monitoring the low-field peak of H-1 with $\underline{f}_1$ while $\underline{f}_2$ was swept through the resonance frequencies of H-2–H-5, either with $\underline{f}_2$ decreasing (fig. 6a–6d) or increasing (fig. 6e–6h). Under favorable conditions, the positive and negative responses [52] in the indor spectra (for example, those at the resonance frequencies of H-2) indicate the progressive or regressive connectivity of the transitions that can be correlated with the lists of progressively and regressively connected transitions provided by the computer program.

The positive responses observed at the resonance frequencies of H-3 (see fig. 6a–6h) indicate the presence of a small, long-range coupling between H-1 and H-3, and, in fact, a more detailed study of the spectrum of fig. 6i has revealed parameters additional to those described in our previous report [20], namely, $\underline{J}_{1,3}$ 0.45, $\underline{J}_{2,3}$ 0.41, and $\underline{J}_{2,4}$ 0.42 Hz.

In this work, a detailed investigation of the influence on homonuclear, indor spectra of the sweep direction and sweep rate of $\underline{f}_2$, and of the power levels of $\underline{f}_1$ and $\underline{f}_2$, has been made. Under certain conditions of sweep rate and power levels, transient nutations [53] of the net, macroscopic, magnetization vector may be observed (see fig. 7). The initial direction of the oscillation also indicates progressive or regressive connectivity, and the rate of decay of the oscillation gives [53] a measure of relaxation times, since the time constant of the decay is equal to the harmonic mean of these times, $\underline{i.e.}$, $(2T_1T_2)/(T_1 + T_2)$.

In other experiments (see fig. 8), the crossover from general Overhauser effects to spin-tickling effects in indor spectra was demonstrated. This crossover occurs because the mechanism by which indor spectra are generated depends on the power level of $\underline{f}_2$. Very low power-levels (see fig. 8c and 8d) cause only transfers of spin population
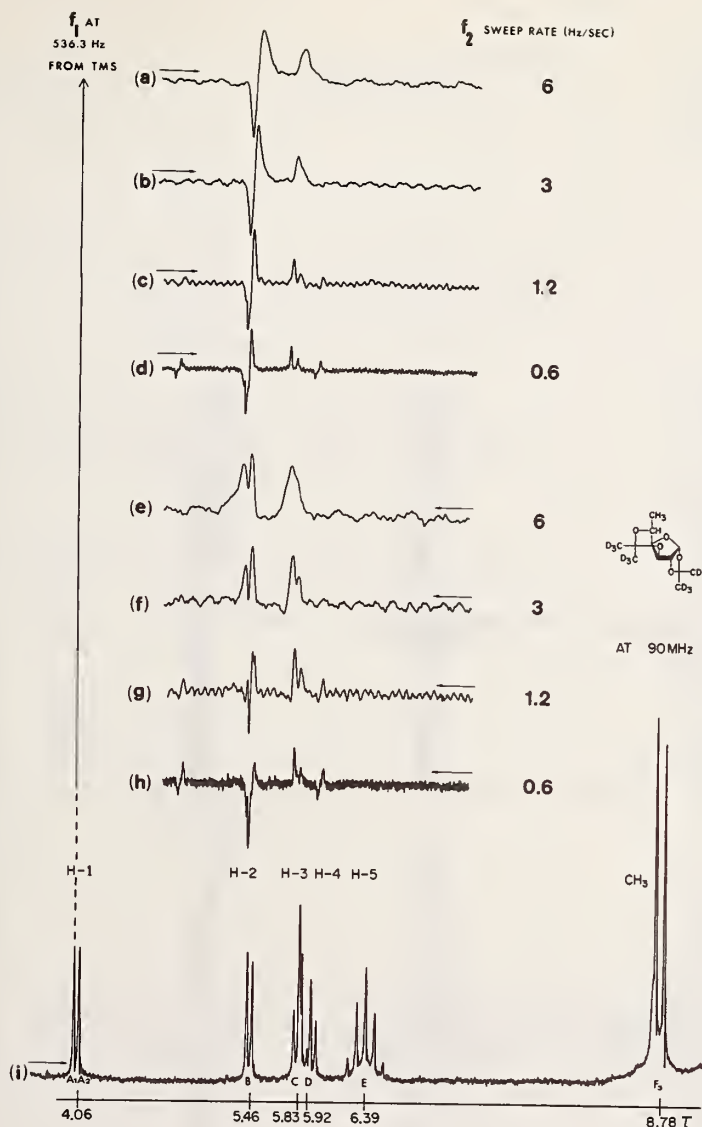
Figure 6. Proton–proton, internuclear, double resonance experiments on 1-d$_{12}$ with f$_2$ decreasing (spectra a–d) or increasing (e–h) at various rates (f$_1$ constant at peak Al of H-1, except for single-resonance spectrum i).
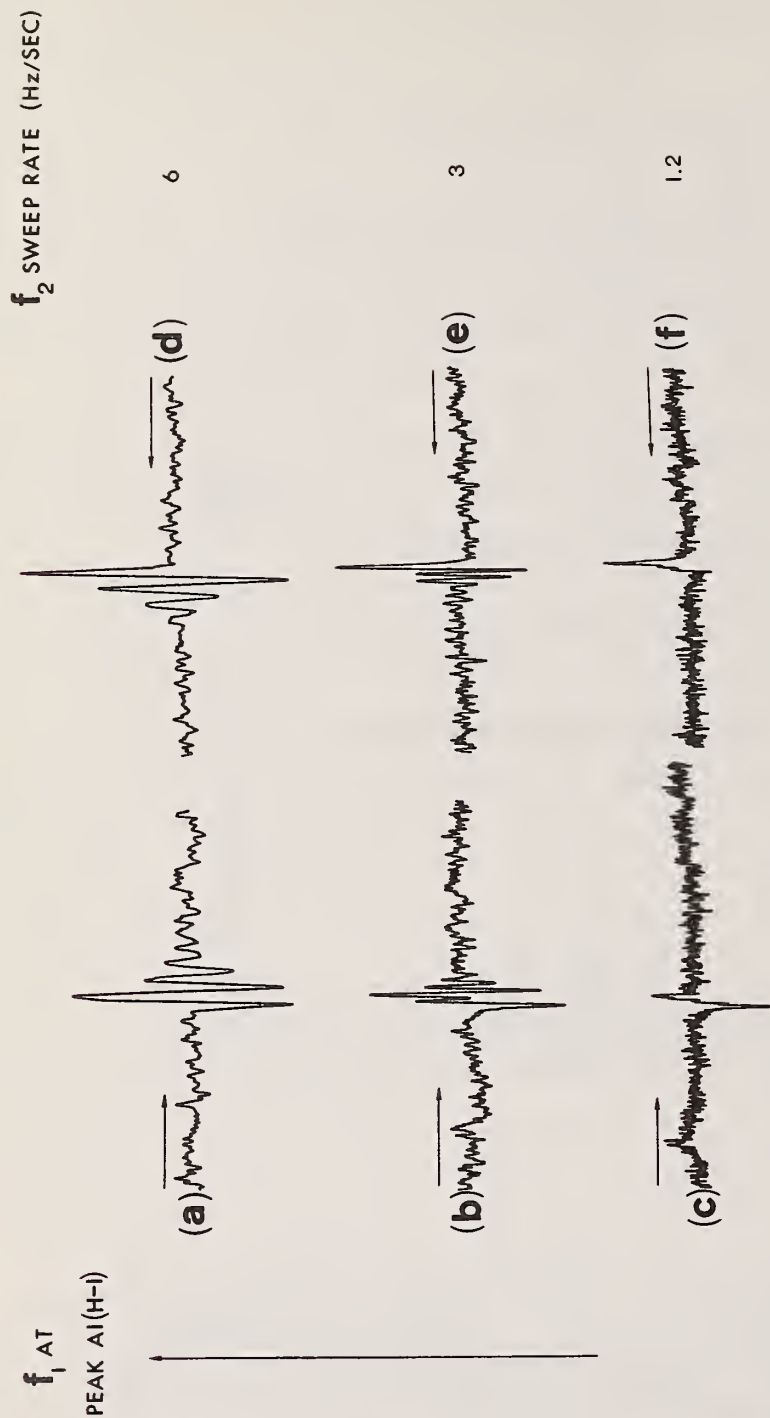
Figure 7. Indor spectra obtained from $\underline{1}$-d_{12} at 90 MHz by monitoring peak Al (H-l) with $\underline{f}_1$ (attenuation, 38) while H-2 was perturbed by $\underline{f}_2$ (attenuation, 44) either decreasing (spectra $\underline{a}$–$\underline{c}$ or increasing (spectra $\underline{d}$–$\underline{f}$) at various rates. Transient nutations are apparent in spectra $\underline{a}$, $\underline{b}$, $\underline{d}$, and $\underline{e}$.
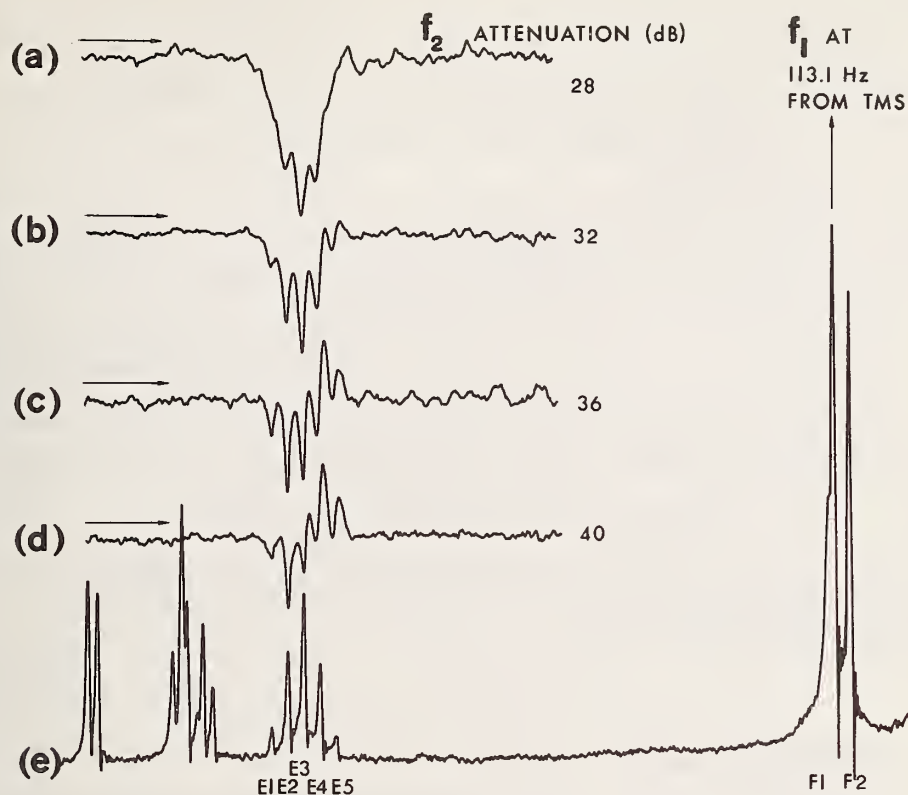
Figure 8.   Proton—proton indor experiments on 1-d₁₂ at 90 MHz with peak Fl (H_3C-6) monitored by a constant f_1, and H-2—H-5 perturbed by a decreasing f_2 applied at various attenuations: (a) 28, (b) 32, (c) 36, (d) 40 dB (down from 0.5 watt), (e) f_1 swept and f_2 off.

between energy levels that result in positive or negative increments to the intensity of the monitored line (general Overhauser effects), whereas higher power-levels (as for fig. 8a and 8b) result in swamping of these effects by spin-tickling effects (negative responses only) due to mixing of spin states.

The structures of compound $\underset{\sim}{1}$-$\underline{d}_{12}$ and its non-deuterated precursor $\underset{\sim}{1}$ have been confirmed by mass spectroscopy. A detailed description of the fragmentation pathways, and of their confirmation by means of the isotopic substitution, together with a review of multiple-resonance techniques and other indor experiments, has been given in a full report which has been accepted for publication [54].

C. <u>Spectroscopic Studies of Derivatives of 6-Amino-6-deoxy-<u>D</u>-glucose-6-¹⁵N</u>

(B. Coxon)

1. <u>Introduction</u>

The synthesis of derivatives of 6-amino-6-deoxy-<u>D</u>-glucose-<u>6</u>-¹⁵<u>N</u> in high yield by reaction of the 6-<u>O</u>-<u>p</u>-tolylsulfonyl or 6-deoxy-6-iodo derivative of 1,2:3,5-di-<u>O</u>-isopropylidene-<u>D</u>-glucofuranose with potassium phthalimide-¹⁵<u>N</u> has been outlined in previous reports [19, 55], as has also the iterative analysis of the p.m.r. spectra (measured at 220 MHz) of the 6-deoxy-6-phthalimido derivatives produced in these reactions [20]. This program for the synthesis and characterization of isotopically labeled, carbohydrate reference-materials of biomedical interest has been continued along the pathways of infrared spectroscopy, mass spectrometry, and heteronuclear, magnetic resonance studies.

2. <u>Infrared Spectroscopy</u>

A brief investigation was made of the isotope effect of the nitrogen-15 isotope on the C—N stretching frequency of 6-deoxy-1,2:3,5-di-<u>O</u>-isopropylidene-6-phthalimido-α-<u>D</u>-

80

glucofuranose (1). This compound shows a strong absorption band at 1400 cm^{-1} (mean of five determinations), whereas its ^{15}N-labeled analog (1-^{15}N) shows it at 1388 cm^{-1}. The isotopic shift of 12 cm^{-1} is considerably smaller than the value (47 cm^{-1}) calculated from the relationship $\nu(C-^{15}N) = \nu(C-^{14}N)\sqrt{(14/15)}$, which is based on Hooke's law and which depends on the assumption that the force constant for each type of carbon—nitrogen bond is the same [56].

3. Mass Spectrometry

Previous studies of the mass spectra of unlabeled amino sugars have been concentrated on their N-acetyl, and N-acetyl-d$_3$ derivatives. For the present work, the availability both of ^{14}N compounds and ^{15}N-labeled derivatives permitted confirmation of pathways of fragmentation by a method alternative to that of deuteration. Thus, molecular fragments that retained the nitrogen-15 atom could be expected to appear at mass units (m/e) one unit greater than those containing the nitrogen-14 atom. This approach was applied to the N,N-phthaloyl derivatives 1 and 1-^{15}N and to the amines 2 and 2-^{15}N derived from them by reaction with hydrazine. The mass spectra of these amines indicate at least four different routes of fragmentation, the most characteristic of which involves scission of the C-5—C-6 bond to give the stable CH$_2$=NH$_2$$^{+}$ ion. Other series of ions were produced from both the amines and their N,N-phthaloyl derivatives by loss of methyl radicals, ketene, acetone, acetic acid, 2-methyl-m-dioxolenium ion, or various combinations of these moieties. The phthalimido group of these derivatives displays its own characteristic fragmentation patterns, including the formation of benzoyl and cyanobenzoyl ions, a dehydro-benzoyl radical cation, a protonated phthalimidomethylene radical ion formed by a McLafferty rearrangement, and a radical ion C$_6$H$_4$$^{+\cdot}$ that has been interpreted as a benzyne ion [57].

81

4. <u>Carbon-13 Magnetic Resonance Studies by Continuous-wave and Fourier-transform Methods</u>

Although our previous studies [19,55] of the proton spectra of 6-amino-6-deoxy-<u>D</u>-glucose-<u>6</u>-^{15}<u>N</u> derivatives at 100 MHz had yielded a selection of ^{1}H—^{15}N coupling-constants that could be expected to help define the applicability of ^{15}N magnetic resonance parameters to the structural and conformational analysis of amino sugars, the measurement of the ^{13}C—^{15}N coupling-constants of these molecules requires direct observation of either ^{13}C or ^{15}N spectra. At the present time, greater progress has been made with ^{13}C n.m.r. than with ^{15}N n.m.r., and so compound 1 and its ^{15}N-labeled analog 1-^{15}<u>N</u> have been studied by both continuous-wave (CW) and Fourier-transform (FT) ^{13}C techniques at 25.2 MHz in order to assist our evaluation of Fourier-transform methods.*

^{13}C spectra of solutions of 1 and 1-^{15}<u>N</u> in benzene were accumulated by CW methods** in 1,024 channels of a signal-averaging computer, whereas spectra of solutions of these compounds in chloroform-<u>d</u> were obtained by FT methods*** in which 4,096 signal-averaged points in the time-domain spectrum were transformed into 2,048 points in the frequency domain. In each method, noise-modulated decoupling of protons was employed to simplify the spectra and provide an enhancement of signal intensity by means of the nuclear Overhauser effect.

By using the FT technique, complete ^{13}C spectra of 1 and 1-^{15}<u>N</u> (see fig. 9) were obtained in one experiment with ∿800 seconds of pulsing, whereas the CW technique required the

---

*By courtesy of L. F. Johnson of Varian Associates.
**Varian HA-100 spectrometer and C-1024 time-averaging computer.
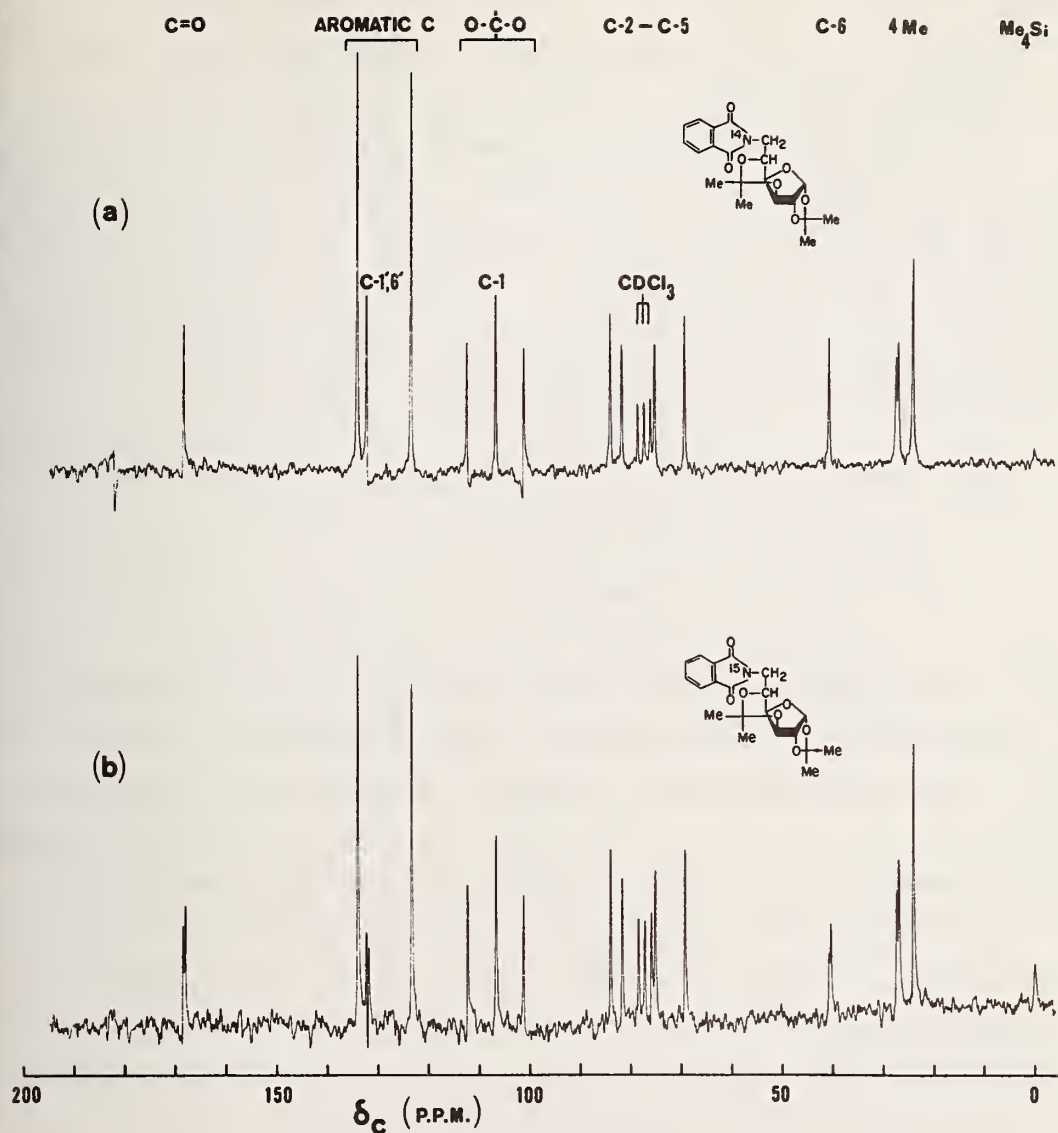***Varian XL-100-15 spectrometer and 620i computer.

Figure 9. Fourier-transform, [13]C n.m.r. spectra of
solutions in chloroform-d, at 25.2 MHz with
proton decoupling: (a) 6-deoxy-1,2:3,5-di-O-
isopropylidene-6-phthalimido-α-D-glucofuranose
(1), (b) 6-deoxy-1,2:3,5-di-O-isopropylidene-6-
phthalimido-α-D-glucofuranose-6-[15]N (1-^{15}N).

division of the ^{13}C spectral region into four zones of
50 p.p.m. each, followed by separate accumulations of 16–289
scans of each zone. For the same accumulation time
($\sim$800 sec) and approximately the same weight of compound
($\sim$0.4 g), the FT technique afforded a signal:noise ratio over
six times that obtained by the CW method, corresponding to a
time-saving factor of about 40. However, correction of
this factor on the basis of division of the CW spectrum into
four zones gives a total time-saving factor of 160.

From fig. 9, it may be seen that three of the carbon-13
resonances appear as singlets for compound $\underset{\sim}{1}$, but doublets
for $\underset{\sim}{1}$-$^{15}\underline{N}$, indicating the coupling constants $^1\underline{J}_{^{13}C-6,^{15}N}$ 9.9,
$^1\underline{J}_{^{13}C=O,^{15}N}$ 12.9 and $^2\underline{J}_{^{13}C-1',^{15}N} = {}^2\underline{J}_{^{13}C-6',^{15}N}$ 11.4
Hz, where C-1´ and C-6´ refer to the aromatic ring.

For the $\underline{N},\underline{N}$-phthaloyl derivative $\underset{\sim}{1}$-$^{15}\underline{N}$ and its $\underline{N}$-acetyl
and $\underline{N}$-(trifluoroacetyl) analogs, the $^1\underline{H}$–^{15}N and ^{13}C–^{15}N
coupling-constants measured in these studies have been used
to assess the degree of s-character in the bonds between thes
nuclei by means of the empirical relationships developed by
Roberts and co-workers [59]. The results indicated that
the polarized, canonical forms of the amide structures of
these compounds are generally more important than the
nonpolarized forms; that is, the ^{15}N atoms of these amides
are closer to sp^2 than to sp^3 hybridization.

5. Heteronuclear, Internuclear, Double- and Triple-resonance Experiments

During the course of the synthetic studies, crystalline
$\underline{N}$-acetyl and $\underline{N}$-trifluoroacetyl derivatives ($\underset{\sim}{3}$-^{15}N and $\underset{\sim}{4}$-$^{15}\underline{N}$,
respectively) of the amine ($\underset{\sim}{2}$-$^{15}\underline{N}$) were prepared, in addition
to the corresponding ^{14}N analogs ($\underset{\sim}{3}$ and $\underset{\sim}{4}$). The N-(trifluo-
roacetyl) derivatives are of special importance, because of
($\underline{a}$) current interest in the use of trifluoroacetyl derivative:
of sugars instead of acetyl or trimethylsilyl derivatives
for gas–liquid chromatography, and ($\underline{b}$) the fundamental

importance of measurements of the magnetic interactions between ^{15}N and ^{19}F.

The p.m.r. spectrum of $\underset{\sim}{4}$ in chloroform-$\underline{d}$ at 90 MHz showed the NH signal as a broad hump having no obvious fine structure, whereas $\underset{\sim}{4}$-$^{15}\underline{N}$ showed two, complex, sub-multiplets separated by $\underline{J}_{^{15}N-H}$ 92.5 Hz. A detailed analysis of this complexity showed that it was due to a combination of long-range coupling ($\sim$0.7 Hz) between the fluorine nuclei and the NH, second-order effects produced by strong coupling of H-5, H-6, and H-6′, and, for compound $\underset{\sim}{4}$, broadening of the NH resonance by the ^{14}N quadrupole.

The ^{19}F n.m.r. spectra of compounds $\underset{\sim}{4}$, $\underset{\sim}{4}$-$\underline{d}$, $\underset{\sim}{4}$-^{15}N, and $\underset{\sim}{4}$-$^{15}\underline{N}$-$\underline{d}$ at 84.7 MHz all showed for the trifluoromethyl group quite narrow resonances that indicated $\underline{J}_{F,^{15}N}$ <0.6 Hz. This coupling is unexpectedly small, but resembles the values $^{3}\underline{J}_{F,F}$ <1 Hz of pentafluoroethyl derivatives for which it has been supposed [60] that the "through bond" contribution to the coupling is attenuated by the (electronegative) fluorine atoms.

The long-range coupling between the fluorine nuclei and NH was confirmed by ($\underline{a}$) irradiation of the fluorine nuclei at a fixed frequency ($\sim$84.7 MHz), whereupon the NH multiplets of $\underset{\sim}{4}$-$^{15}\underline{N}$ sharpened up, and ($\underline{b}$) indor experiments in which $\underline{f}_2$ was swept through the fluorine region while one of the stronger lines of the NH multiplet was monitored by $\underline{f}_1$. In order to assist the analysis of these complex spectra, other indor experiments were conducted in which $\underline{f}_2$ was swept through the ^{15}N resonance frequency at $\sim$9.12 MHz; and also, internuclear triple-resonance (intripler) experiments in which $\underline{f}_3$ was swept at $\sim$9.12 MHz while a fixed $\underline{f}_2$ was applied at $\sim$84.7 MHz in order to decouple the fluorine nuclei from the NH proton. For most of the power levels of $\underline{f}_1$ and $\underline{f}_2$ or $\underline{f}_3$ investigated, positive responses of the NH peak were observed (see fig. 10b) as
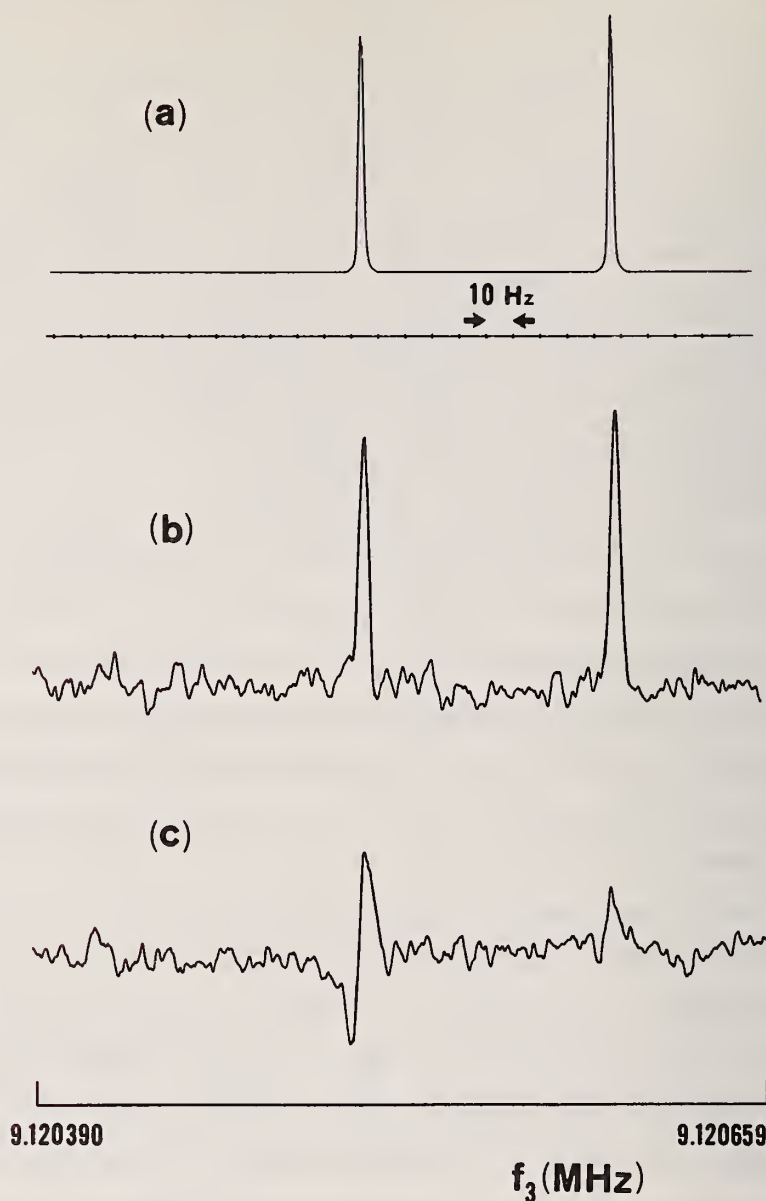
85

Figure 10. ^{15}N-related n.m.r. spectra of 6-deoxy-1,2:3,5-di-O-isopropylidene-6-(trifluoroacetamido)-α-D-glucofuranose-$6^{15}N$ ($4-^{15}N$) in chloroform-d: (a) computed, theoretical, ^{15}N n.m.r. spectrum; (b) ^{15}N n.m.r. spectrum obtained indirectly from heteronuclear, intripler experiment in which a ^{19}F decoupled NH proton peak was monitored by f_1, at ∿90 MHz, while the ^{15}N frequency (f_3) was swept; (c) the same, with f_1 at lower power.

86

f_2 or f_3 was swept through the resonance frequencies of each group of ^{15}N transitions. The advantage of the intripler experiment was that positive responses having 12 percent greater amplitude (on average) were obtained in the presence of ^{19}F decoupling than in its absence, due, apparently, to the fact that the decoupling gave a sharper NH peak to be monitored by f_1. It may be seen that the intripler spectrum shown in fig. 10b is a replica of the theoretical, ^{15}N spectrum computed for 4-$^{15}\underset{\sim}{N}$ (see fig. 10a). These results are·unusual, and are in agreement neither with the negative and positive responses expected from considerations of connectivity as the ^{15}N frequency was swept from low to high values, nor with the negative peaks expected from spin-tickling effects. It seems that, for most of the conditions employed, general Overhauser and spin-tickling effects are dominated by a positive, intramolecular, nuclear Overhauser effect due to magnetic dipole—dipole interaction between the ^{15}N and proton nuclei. Full numerical data and details of the experimental methods are given in a paper that has been accepted for publication [61].

D. Cyclic Polyhydroxy Compounds

(A. J. Fatiadi)

1. (p-Bromophenyl)osotriazoles from Inositols

In continuation of studies on cyclitols, the products resulting from treatment of three inosose phenylosazones with aqueous bromine have now been examined. As expected, the products were (p-bromophenyl)osotriazoles ($\underset{\sim}{1}$, $\underset{\sim}{2}$, and $\underset{\sim}{3}$), albeit they were isolated in only 28—42 percent yield.
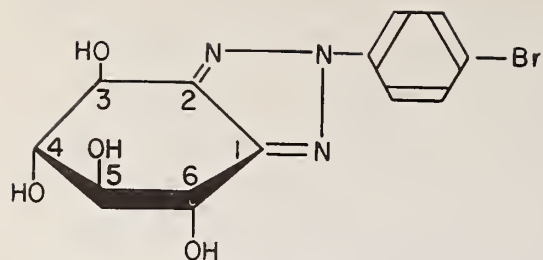
D

1
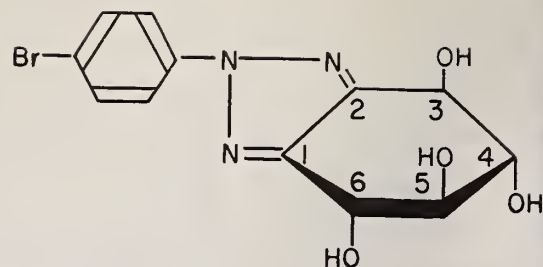
L

2
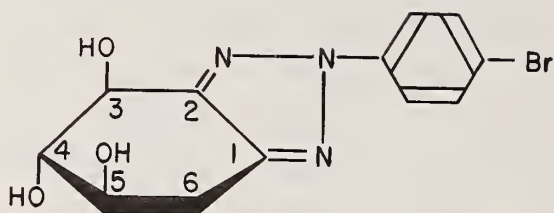
3

In addition to preparation of their acetates or benzoates, the phenylosotriazoles were oxidized with periodate, to afford new dialdehydes. A note on this work has been accepted for publication [62].

3. GENERAL ORGANIC CHEMISTRY

(A. J. Fatiadi)

A. Oxidative Cleavage of Aromatic Azines with Periodic
   Acid: E.S.R. Evidence for the Liberation of
   Nitrogen Dioxide

As part of a continuing interest in non-Malapradian
reactions of periodic acid, a number of aromatic and
heterocyclic azines (1a-1e) have been treated with periodic
acid at room temperature. Brisk evolution of nitrogen was
observed in all cases, indicating the lability of the
hydrazino moiety (⟩C=N-N=C⟨) of the azines towards this
reagent. Extraction of the reaction mixture gave the
parent aldehyde (or ketone) in over 95 percent yield,
indicating that the oxidative cleavage was complete.
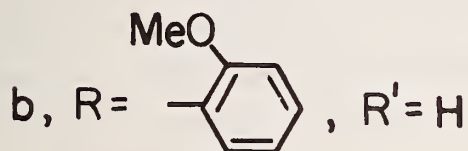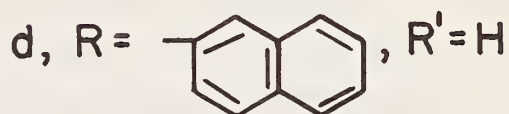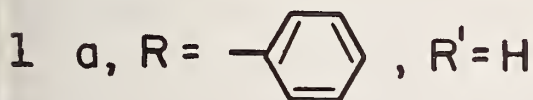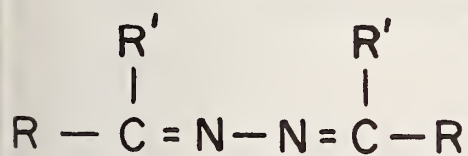
In a typical experiment, a solution of benzalazine
(1,4-diphenyl-2,3-diazabuta-1,3-diene) (1a) (1.04 g, 5
mmol) in glacial acetic acid or N,N-dimethylformamide
(15 ml) was stirred with an aqueous solution of para-
periodic acid (2.3 g, 3 ml, 10 mmol) at room temperature
for 15 min. An exothermic reaction ensued, and brisk
evolution of nitrogen commenced immediately after mixing.
The mixture was treated with a few drops of 10 percent
aqueous sodium hydrogen sulfite to remove iodine (and
any excess of the oxidant), acidified with hydrochloric
acid if necessary, and extracted with ethyl ether (4 X 10 ml),
to give benzaldehyde in 96 percent yield.

Similarly, by the use of a 1:2 molar ratio of substrate
to oxidant, azines 1b-1e were converted into the parent
aldehydes or ketones in over 95 percent yield.

The formation of nitrogen may arise through collapse
of an association complex initially formed between periodate
dianion and a resonance structure of the azine 1a. The
structure of the complex and the pathway of formation of
nitrogen are shown in fig. 11.

Examination of the association complex (fig. 11) showed
that the azine had already suffered a two-electron oxidation
(formation of two bonds), and the question was whether to
depict a transition complex with iodine(VII) or with iodine(V)
Our statement that the formation of a complex (fig. 11) is a
symmetry-allowed reaction, as predicted from MO symmetry rules,
was based on the assumption that iodate ion I(V) should be
depicted. However, the e.s.r. evidence regarding the action
of both oxidants (H_5IO_6 and HIO_3) on azines, particularly
a low-field peak at 1.864 kG due to a binuclear, triplet-
state species that is observed only for periodic acid, made
it difficult to conceive the existence of a dimer species
for the iodic acid (or its ion) in solution. Consequently,
the formation of such a complex may involve, as a single step,
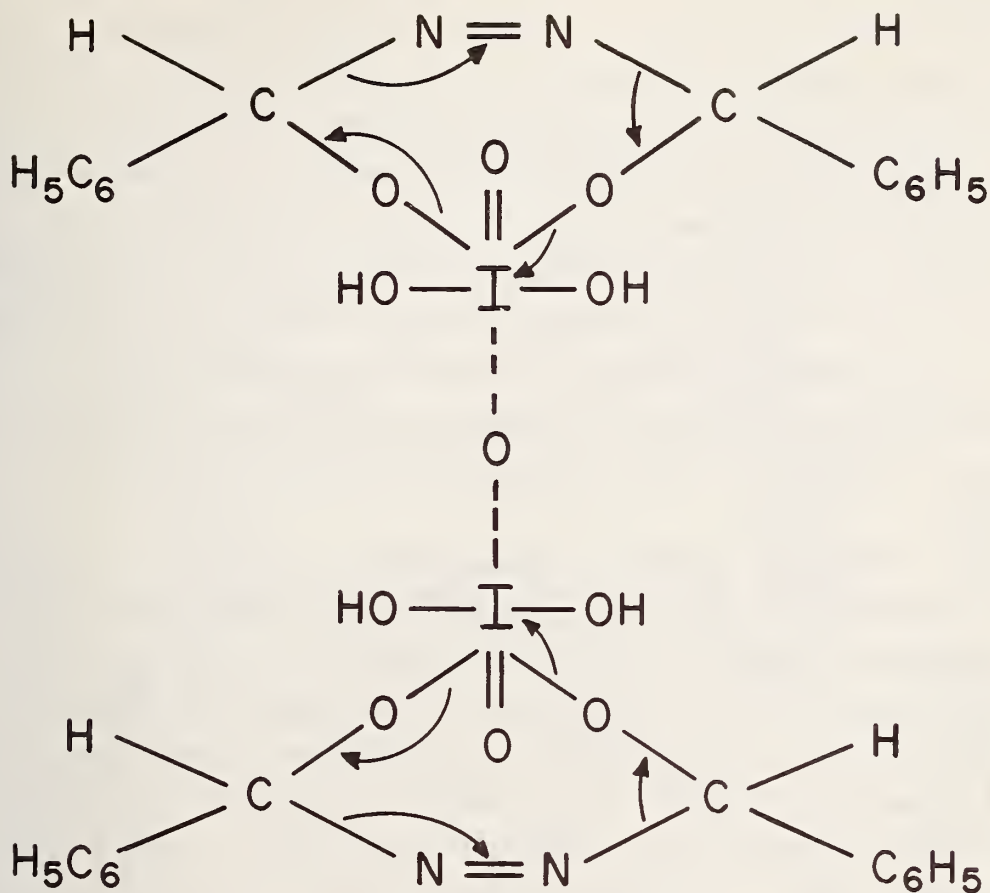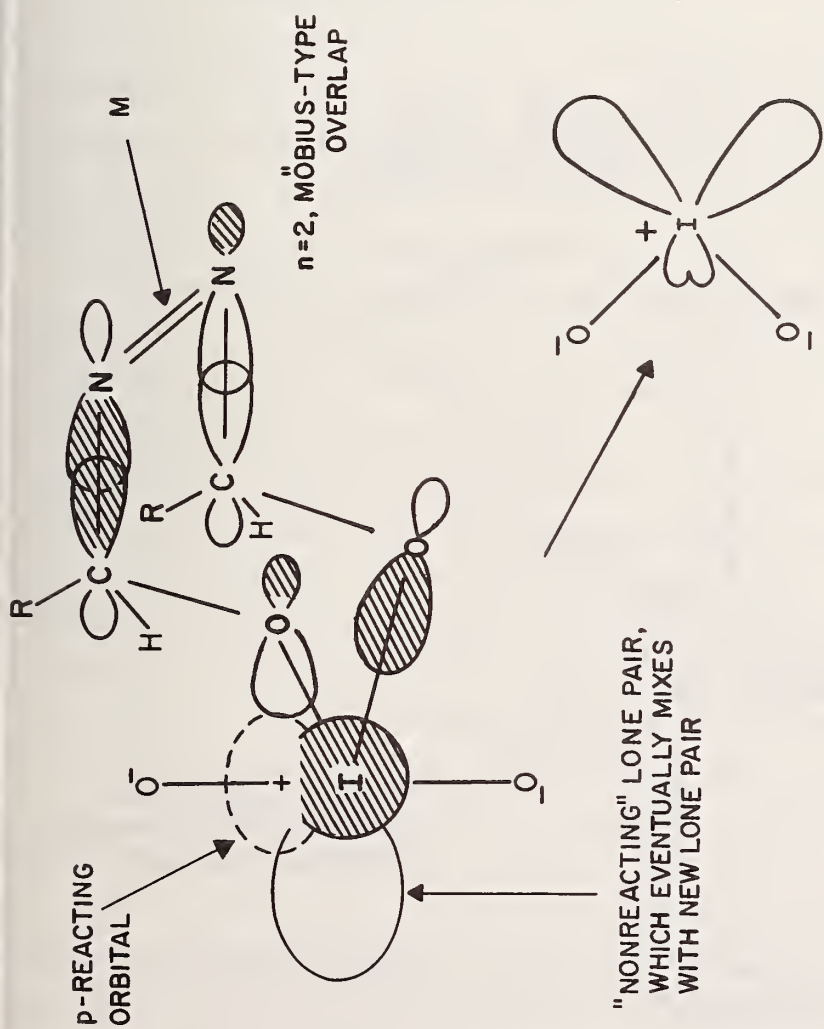the addition of IO_4^- (or its hydrate) across the conjugated

Figure 11. Proposed structure of the association complex between periodate dianion and a resonance structure of azine 1a.

azine system, to give an iodate I(V) complex; this view is in agreement with that of another worker [63].

The molecular-orbital overlap in the transition state, particularly in the formation of the complex (fig. 11) can be treated either by symmetry or topology. The symmetry rules of Woodward and Hoffmann [64] require knowledge of the molecular orbitals of the reagents or products; the alternative treatment by Zimmerman [65], using a topological approach to the orbital levels of the transition state, gives rise to the same predictions as the Woodward—Hoffmann treatment, but it does not require a correlation of the molecular orbitals of the reactants with those of the products.

The Zimmerman method [65] requires the classification of the transition state as either "Hückel-like," in which all overlapping pairs of reacting orbitals are bonded, or "Möbius-like," in which one, or an odd number of, anti-state bonding overlap(s) must occur. In a Hückel transition state, $4n$ + 2 electrons form a closed shell (giving it stability), whereas $4n$ electrons are required for stable, closed shells in a Möbius state. It will be noted that $4n$ electrons are involved in the azine oxidation with periodic acid (fig. 11) Thus, an "allowed" reaction may have either a Hückel or Möbius type of transition state, provided that the correct number of electrons is available to stabilize that transition state [66].

One possible way for an "allowed" reaction that should include an allowed decomposition of iodine(V) in the iodate I(V) analog of the complex (fig. 11) is depicted in scheme 1. The immediate difficulty, however, of imagining an addition step involving iodate ion I(V) to benzalazine to give the complex (fig. 11) can be overcome by the possible pathway shown in scheme 2. A communication on this topic has been published. [67].

M

n=2, MÖBIUS-TYPE OVERLAP

p-REACTING ORBITAL

"NONREACTING" LONE PAIR, WHICH EVENTUALLY MIXES WITH NEW LONE PAIR

IO_2^+ WITH TWO LONE PAIRS IN sp^3-TYPE ORBITALS

$R = C_6H_5$

IO_2^+ IS ASSUMED TO HAVE THE SAME STRUCTURE AS ICl_2^+

THE I (V) REACTANT MUST THEREFORE BE 4-COORDINATE (AS $IO_2F_2^-$)

SCHEME I

93

n = 2, MÖBIUS-TYPE OVERLAP

p – REACTING ORBITAL

"NONREACTING ORBITAL"
ON 3-FOLD AXIS OF IODATE

TWO sp³d LONE PAIRS
ON EQUATOR OF TRIGONAL
BIPYRAMID (AS BrF₃)

R = C₆H₅

FOLLOWED BY REACTION WITH I(VII) TO REGENERATE I(V)
AS REQUIRED FOR SCHEME I

SCHEME 2

94

B. Evidence for Adsorption as the First Step in the
Solid-state Oxidation of Benzenehexol with Active
Manganese Dioxide

Evidence has been acquired of an unusual adsorption
on the surface of manganese dioxide, as the first step in
the solid-state oxidation of benzenehexol (1) to give 4
(see scheme 3); a possible pathway in the conversion of 1
into 4 is also discussed and a structure for the solid
oxidant is suggested.

1. Possible Structure of the Precipitated Manganese
Dioxide

The adsorptive ability of manganese dioxide has been
thoroughly studied; for example, its adsorption of water or
benzene vapors, hydrogen, organic substrates from solution,
and inorganic cations (Ba^{2+}, Mn^{2+}, or Na^+); an example of
adsorption of an organic solid on a solid is now described.

The mechanism of oxidations with manganese dioxide
undoubtedly involves a process typical of heterogeneous
reactions, and the difficulties encountered in the study of
these reactions may involve the structure of the oxidant,
particularly the relationship of its surface-active sites
toward a substrate (quadrupole—dipole electrostatic
interaction [68]). The amorphous character of the precipitated
manganese dioxide is shown in the X-ray diffraction spectra
A-1 and B-1 (fig. 12), and this character is in accordance
with earlier observations [69,70]. To our surprise, however,
the oxidant prepared in deuterium oxide instead of water

$$MnSO_4 + KMnO_4 + D_2O \text{ (acidic, 80 °C)} \rightarrow MnO_2$$
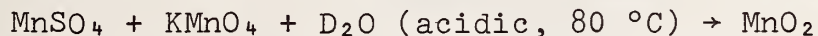
was a dark, crystalline product having the pattern shown in
C-1 and D-1 (fig. 12). The structure of this compound
resembled that of γ-MnO_2, as shown by comparison of their
interplanar spacings and intensities. In order to shed more
light on its structure, a sample of active manganese dioxide
(300 mg) was decomposed by stirring it with aqueous
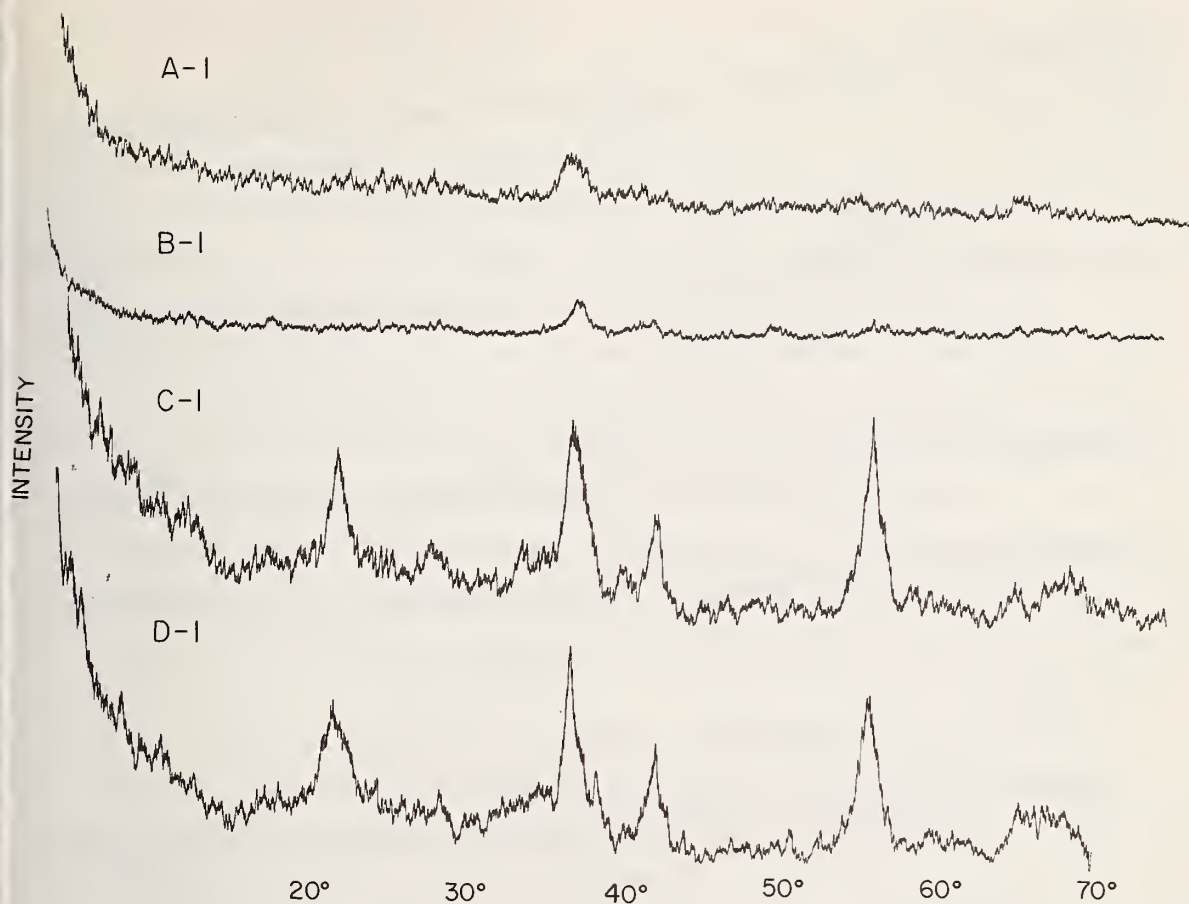
95
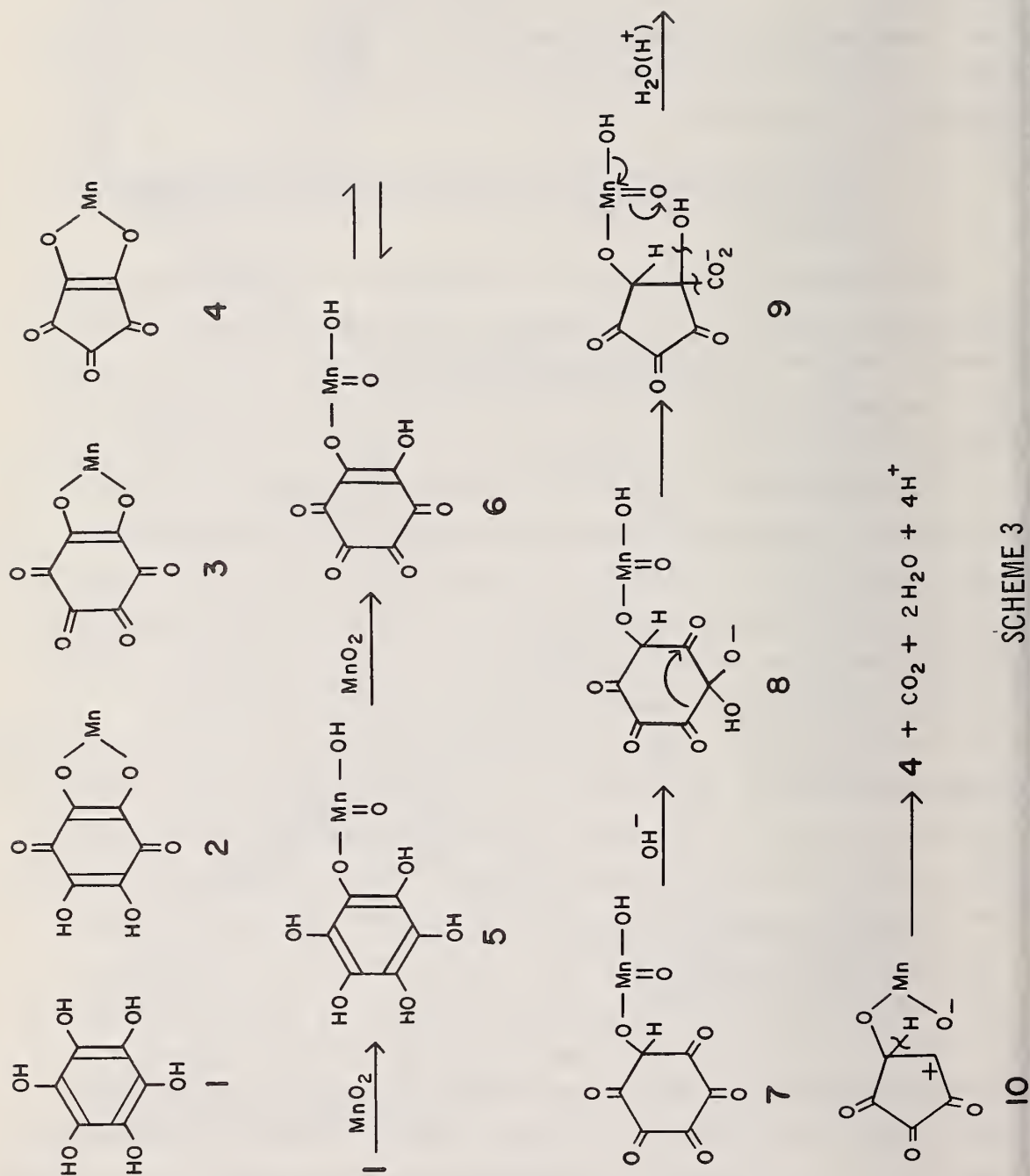
SCHEME 3

Figure 12. X-Ray powder diffraction pattern of manganese
dioxides (CuKα radiation): A-1, precipitated
manganese dioxide dried in vacuo at room
temperature; B-1, sample A-1 dried in an oven
at 125 °C; C-1, manganese dioxide prepared in
deuterium oxide as described in the text (dried
in vacuo at 100 °C); D-1, sample C-1 exposed to
air for 3 months.

97

hydrochloric acid (3 $\underline{M}$, 20 ml) at room temperature for 24 h
in a hood (evolution of chlorine); the dark residue
(50—60 mg) that resisted decomposition was crystalline.
The isolated oxide contained manganese of the lower valency,
and its interplanar spacings resembled [71] that of $MnO_{1.88}$.
Moreover, its infrared spectrum showed little or no adsorption
in the region for hydroxyl bands, in comparison to that of
the initial manganese dioxide prepared in water (OH band
at 3380 cm^{-1} water of hydration at 1625 cm^{-1}) or the
manganese dioxide prepared in deuterium oxide (OD band at
3350 cm^{-1}, deuterium oxide of "hydration" at 1535 cm^{-1}).
A crystalline oxide containing manganese of lower valency
was also obtained on partial decomposition of the active
manganese dioxide with aqueous hydrogen peroxide at room
temperature.

The e.s.r. measurements of the oxidant (A, fig. 13)
and of the complex (D, fig. 13) showed, for each, a broad,
one-line peak which can be explained as being due to a
dipole—dipole interaction between polycrystalline manganese
dioxide aggregates. Alternatively, the line broadening
observed may be due to interaction of an unpaired electron
with a proton of a neighboring water molecule, as has been
reported [72] for certain coordination complexes (in this
case, precipitated manganese dioxide). The situation is
comparable to the change in structure of the oxidant from
amorphous to crystalline when hydrogen is replaced by
deuterium (see fig. 12), and lessening in the line width
for the deuterated oxidant is expected. On the basis of
this evidence and the e.s.r. spectrum of the oxidant,
precipitated manganese dioxide may contain a locked, water-
associated chain as depicted; this structure provides
important active sites of low electron-density on the surface
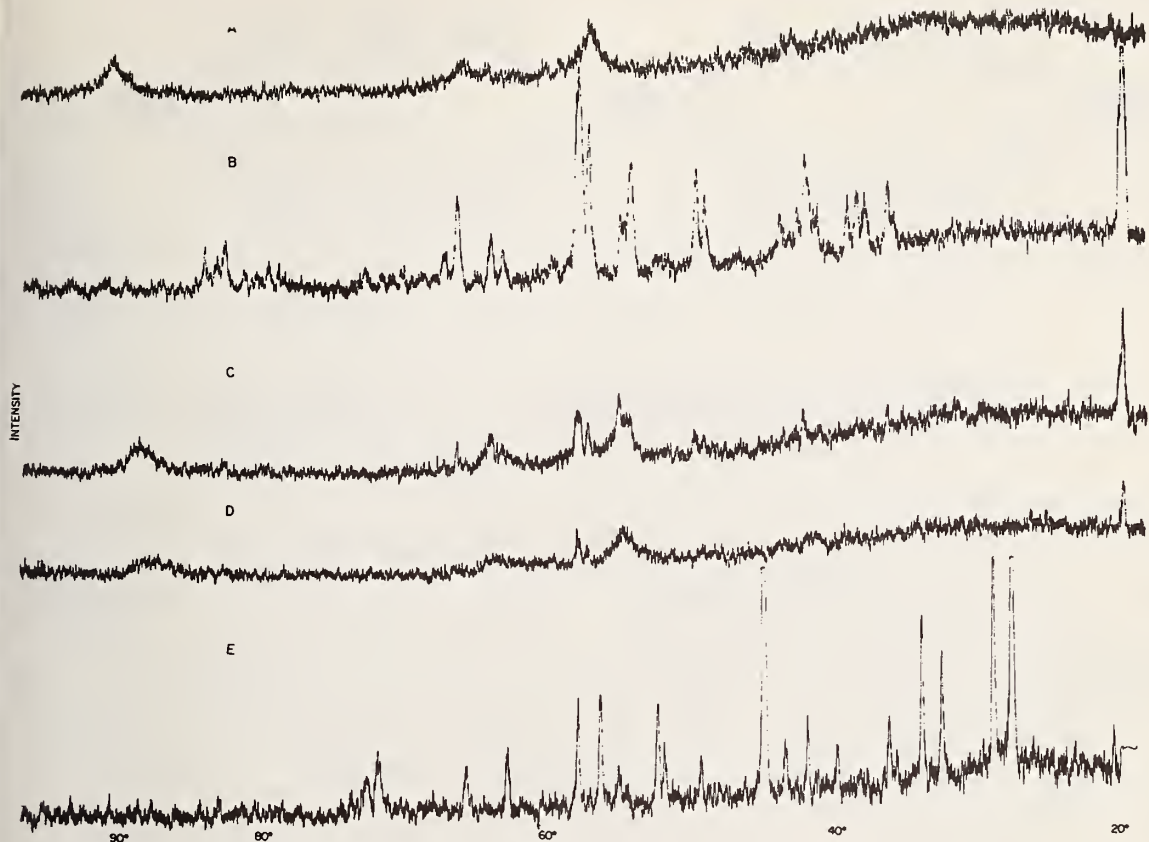of the solid oxidant [68] and potentially labile hydroxyl
groups.

Figure 13.   X-Ray powder diffraction pattern (Cr-Kα radiation)
of aliquots, on mixing of 1:6 (w/w) benzenehexol
(1) and manganese dioxide. (The 1:6 ratio is
for substrate to oxidant.) A, precipitated
(amorphous) manganese dioxide; B, 30 sec,
structure shown is primarily due to 1; C, 50 min,
∿45% of 1 adsorbed; D, 100 min, ∿97%~of 1 adsorbed,
complex D̃; and E, manganese croconate trĩhydrate,
purified product extracted from D.
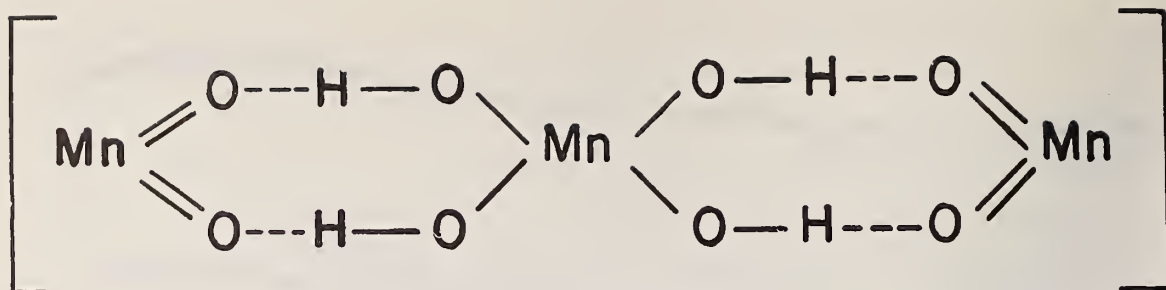
2. Adsorption of Benzenehexol (1) by Manganese Dioxide

A 1:6 (w/w) mixture of finely ground 1 [73] and
manganese dioxide was mechanically shaken at room temperature,
and the rate of disappearance of 1 was monitored by use of
an X-ray powder diffractometer. Fig. 13 shows the background
spectrum A (amorphous manganese dioxide); the decrease in
the concentration of 1 with time is seen in spectrum B
(30 sec; the structure shown is primarily due to 1) and in
spectrum C (50 min; ∿45% of 1 adsorbed); in spectrum D,
at 100 min, ∿97% of 1 is adsorbed. The high affinity of
manganese dioxide for 1, which seems to be an example of an
irreversible adsorption (none of 1 could be extracted from
the complex), is typical of a chemisorption [74]; also, the
adsorption can partially be explained as being due to the
formation of a π-complex between 1 and the oxidant [75].
The formation of an ionic bond (-O-Mn-O-) is, however, evident
from the isolation of 4.

The amorphous pattern shown in spectrum D (fig. 13) was
almost identical with that of manganese dioxide (spectrum A);
however, an electron-diffraction probe of the complex at
this stage showed indeed, a crystalline pattern, thus proving
that adsorption of 1 by the oxidant is exclusively a surface-
interaction process.

Extraction of this complex with water, and gradual
concentration of the extract, yielded, first, a small
quantity of white crystals, identified as manganese oxalate

100

by X-ray diffraction, and then greenish crystals of manganese croconate (4) (E, fig. 13).

The measurements of magnetic susceptibility of a 1:6 (w/w) mechanical mixture of 1 and manganese dioxide at room temperature showed little appreciable change (from 2254 to 2262 x 10^{-6} cm³/mol) after 5, 30, 60, 100, and 1,000 min; the final value observed is comparable to that reported for precipitated manganese dioxide (2280 x 10^{-6} cm³/mol).

3. Reaction Mechanisms Involved in Oxidation of
    Benzenehexol (1) to Croconic acid (4) by
    Manganese Dioxide

Possible chemical transformations of 1 into 4 are shown in scheme 3; this conversion may involve the manganese ester intermediate 5, an oxidation process to give intermediates 6 and 7, and benzilic acid-type rearrangements of intermediates 8 and 9 to give, via carbonium ion 10, product 4. Whereas conversion of 7 into 4 requires participation of a strong nucleophile (hydroxyl ions) (see also, the correlation of increase in yield of 4 in alkaline media), the steps from 1 to 7 and the presence of manganese oxalate in the product (probably arising from carbon-carbon cleavage of 1) can only be reconciled by invoking radical participation.

Refluxing (60 min) of a mixture of 1 (1 g), manganese dioxide [76] (12 g), and dry benzene (200 ml, Dean-Stark trap) gave 0.25—0.40 ml of water. Crude 4 (250 mg) was obtained by extraction of the reaction mixture with water. After the reaction, analysis of the benzene (by g.l.c.) revealed the presence of a trace of phenol, which could have been formed by collision of hydroxyl radicals (derived from manganese dioxide) with the solvent; also, the trapped water indicates the participation of hydroxyl radicals [77].

Assuming that our hypothesis is correct, consumption of one equivalent of active oxygen present in manganese

101

dioxide corresponds to two hydroxyl radicals, as shown for
a certain hydrated form of it; and this pathway would provide

$$MnO(OH)_2 \rightarrow MnO + 2 \cdot OH \rightarrow 2\ O^* + H_2O + Mn^{2+} + 2\ e$$

species that could either (<u>a</u>) abstract hydrogen atoms or (<u>b</u>)
donate hydroxyl radicals. This mechanistic approach is
analogous to those proposed for the oxidation of alcohols
by nickel peroxide [78], with the difference that any para-
magnetic species formed on the surface of manganese dioxide
is probably consumed immediately, as, otherwise, any species
having free spins would have been detected by the balance
used in magnetic-susceptibility measurements. In this
alternative aspect, trapping of free radicals in solution
may furnish additional evidence to clarify the mechanism
of the oxidation.

Thus, conversion of 1 into 4 by manganese dioxide
probably proceeds by a concerted mechanism involving ionic
and free-radical pathways. This work has been published [79].

C. Stable Radical-anions Derived from Glyoxal
    Bis(phenylhydrazones)

In the series of stable hydrazyl radicals, a neutral
radical, namely, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and
its analogs are the best known and the most thoroughly
studied [80,81]; recently, e.s.r. studies established the
existence of stable, hydrazinium radical-cations in colored
solutions of tetraphenylhydrazine dissolved in strong acids
[82]. The preparation and some of the properties of some
new, stable, hydrazyl radical-anions are now described.

Treatment of a solution of glyoxal bis(phenylhydrazone)
(1a) in methyl sulfoxide (Me$_2$SO) (10^{-2} to 10^{-3} $\underline{M}$, $\lambda_{max}^{Me_2SO}$
382 nm, $\varepsilon_{m\underline{M}}$ ~49) with a strong base (5% aqueous potassium
hydroxide or potassium tert-butoxide in Me$_2$SO) and a trace
of oxygen produced a deep violet-purple solution exhibiting
bands at λ_{max} 455 and 562 nm having rapidly changing
intensity. E.s.r. examination of the solution revealed that
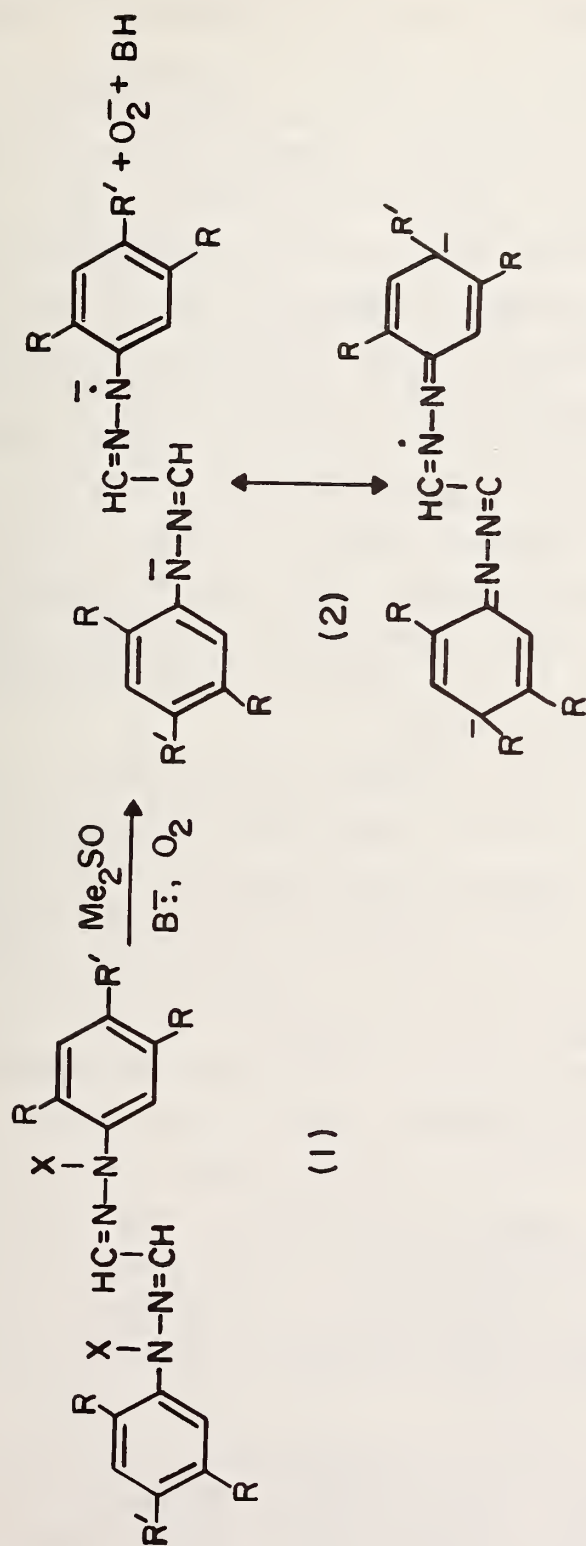
(1)

(1a) R = R' = X = H
(1b) R = R' = H, X = Me
(1c) R = R' = H, X = OCPh
(1d) R = X = H, R' = Cl, Br
(1e) R = X = H, R' = NO_2
(1f) R = R' = H, R = m–NO_2

(2)

(2a) R = R' = H
(2b) R = Cl, R' = H
(2c) R = H, R' = Br
(2d) R = R' = H, R = m–NO_2

the deep-purple color, and, consequently, a bathochromic
shift in the visible spectrum, is partly associated with
the presence of a paramagnetic species having the hyperfine
structure shown in formula 1 (A and B).

Cooling and rapid filtration of the concentrated,
alkaline solution from 1a yielded a somewhat air-sensitive,
purple-violet, paramagnetic dipotassium salt (2 ↔ 2a). From
the fact that neither the N-methyl nor the N-benzoyl derivative
derivative of glyoxal bis(phenylhydrazone) (1b and 1c,
respectively) showed either a color change or the presence
of paramagnetic species following treatment with base, it
was concluded that the purple radical-anion from 1a must
arise following the abstraction of acidic imino protons
(X in 1a) by base. The dianion character of the radical
anion (2 ↔ 2a) was deduced from the fact that methylation
or benzoylation of the latter gave the di-N-methyl (1b) or
di-N-benzoyl (1c) derivative of glyoxal bis(phenylhydrazone)
in 45 and 60% yield, respectively.

The contribution of the resonance form (2 ↔ 2a) to the
stability of the radical anion from 1a was deduced from a
study of radical anions derived from substituted bis(phenyl-
hydrazones). The most stable radical-anions were obtained
from (a) the unsubstituted glyoxal bis(phenylhydrazone) 1a,
(b) those having the para position in the benzene ring
unsubstituted (for example, the 2,5-dichloro derivative, 2b),
or (c) those in which the example, para position was substitu-
ted with an electron-withdrawing group, for example, the
p-nitro derivative 1e. Derivatives having electron-donating
groups on the phenylhydrazino group (for example, the p-chloro
or p-bromo derivatives 1d) produced, on treatment with base,
little or no bathochromic shift and gave little or no e.s.r.
signal.

These observations are in agreement with the substituent
effect proposed by Walter [83], in which it was considered
that electron-withdrawing groups (e.g., p-NO$_2$) enhance the

104

stability of the hydrazyl radical by delocalization of an unpaired electron. It should be mentioned, however, that, in contrast to neutral and cationic hydrazyl radicals having p-Cl or p-Br substituents [80], the corresponding anion-radical of type 2c was not observed following treatment of the glyoxal p-Cl- or p-Br-substituted bis(phenylhydrazones) 1d with base. Formation of a radical anion (2 ↔ 2a) from the bis(phenylhydrazone) 1a in the presence of oxygen and base appears to be a straightforward example of electron transfer; similar electron transfer occurs in the formation of radical anions from formazans [84]. The bis(phenylhydrazone) 1a gave the e.s.r. spectra shown in fig. 14. The 15 lines in spectra A and A-1 (aqueous potassium hydroxide) had intensities of ∿1:2:6:10:14:18:22:24:22:18:14:10:6:2:1. Structure A is a centrosymmetric, resonance-stabilized, radical anion having two different pairs of nitrogen atoms (N=CH and N-Ph); e.s.r. spectrum (A and A-1, fig. 14) can be explained by assuming interaction of the unpaired electron with two pairs of equivalent nitrogen atoms; this spectrum is consistent with a^N = 4.95 and 4.95 G, a^H = 4.95, 4.95, 2.35, and 2.35 G. Spectrum B (fig. 14) (prepared with anhydrous potassium tert-butoxide in methyl sulfoxide) is consistent with a^N = 5.70 and 5.70 G; a^H = 5.70, 5.70, 1.41, 1.41, 0.70, and 0.70 G. The radical intermediate may be represented as a resonance-stabilized, acyclic structure (trans-cis structures 3 ↔ 3a); however, the cyclic 1,2,3,4-tetrazine structure (4 ↔ 4a) cannot be excluded completely.

A symmetrical radical-anion 2b derived from glyoxal bis[(2,5-dichlorophenyl)hydrazone] gave the e.s.r. features shown in fig. 14C; this can also be explained by assuming interaction of the unpaired electron with two pairs of equivalent nitrogen atoms, a^N = 6.35 and 6.35 G; a^H = 6.35, 6.35, 1.65, 1.65, 0.82, and 0.82 G.

A paper describing some properties of these stable radicals has been accepted for publication.
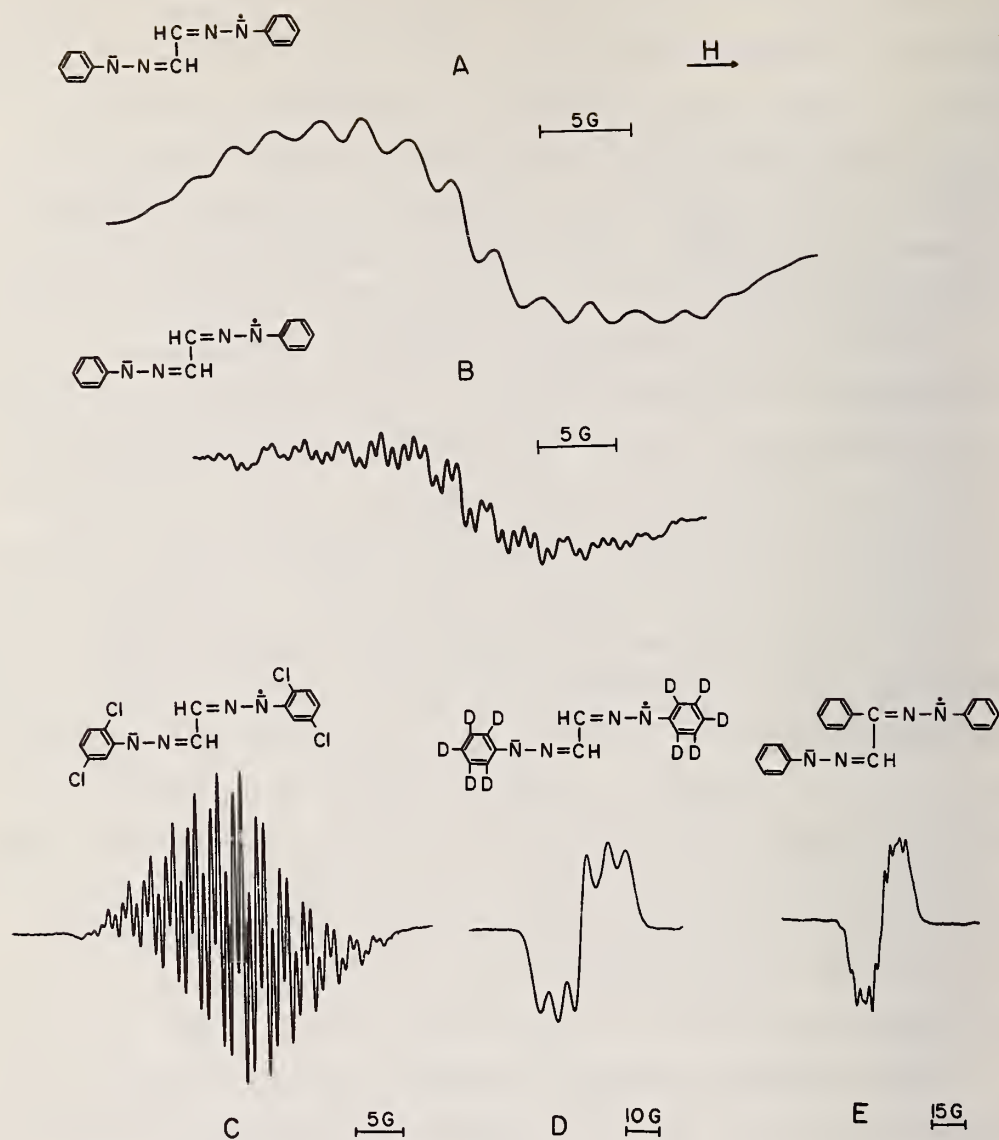
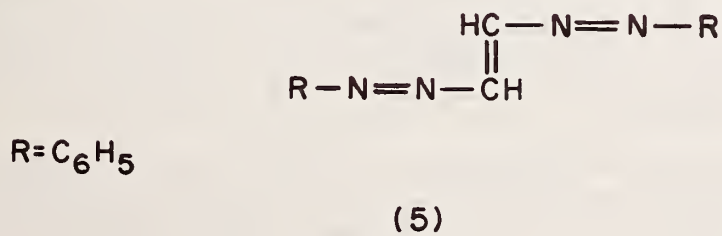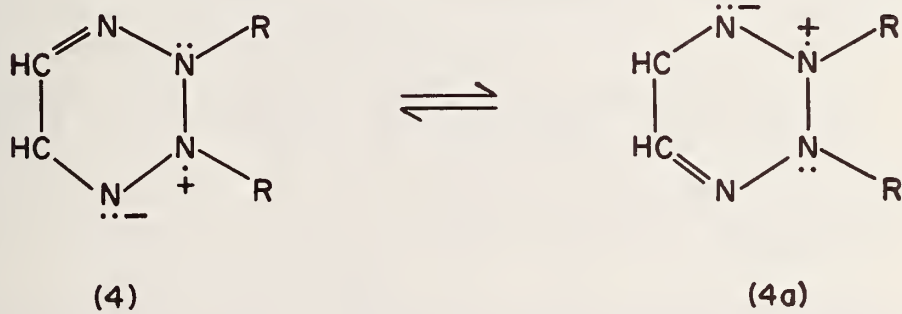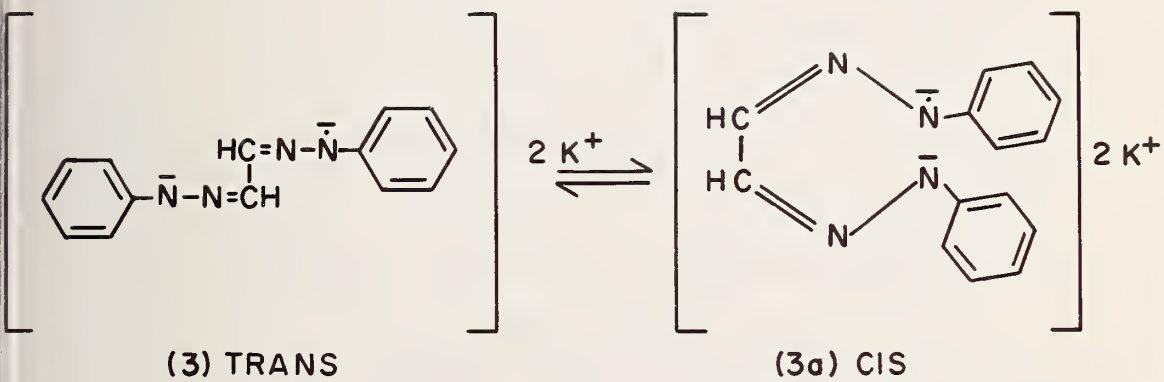Figure 14. Radical-anions from glyoxal bis(phenylhydrazones):
A, from 1a (3.2 mM, in Me₂SO–5% aqueous KOH); B,
from 1a (2.8 mM, in Me₂SO–KOBuᵗ; C, from 2,5-
dichloro derivative of 1a (3 mM, in Me₂SO–KOBuᵗ);
D, from 1a completely deuterated on the benzene
ring (5 mM, in Me₂SO–KOBuᵗ); and E, from
phenylglyoxal bis(phenylhydrazone).

(3) TRANS   (3a) CIS

(4)   (4a)

$R = C_6H_5$

(5)

107

A. <u>Personnel Listing</u>

R. Schaffer, Section Chief

R. S. Tipson

B. Coxon

A. J. Fatiadi

R. F. Brady, Jr.

W. H. McCurdy, Jr.

W. J. Rossiter, Jr. to 7-13-70

B. F. West (Mrs. A. M. Tejeda) to 5-21-71

A. Cohen

B. A. Johnson

E. C. Douglass, Guest Worker (1970, 1971)

E. Hohertz, Guest Worker (1970)

J. James, Guest Worker (1970, 1971)

B. <u>Publications</u>

NBS Technical Note 547. Organic Chemistry Section.
7/1/69 - 6/30/70.
  Edited by R. Schaffer (November, 1970).

Isotopic Methods [in Carbohydrate Chemistry].
  R. Schaffer, in "The Carbohydrates: Chemistry and
  Biochemistry," edited by W. Pigman and D. Horton,
  Vol. 2B, 1970, pp. 765-776.

The Conformations of 1,2:4,6-Di-<u>O</u>-benzylidene-α-<u>D</u>-
glucopyranose Derivatives.
  B. Coxon, Carbohyd. Res. <u>14</u>, 9 (1970).

Cyclic Acetals of Ketoses. Part III. Re-investigation
of the Synthesis of the Isomeric Di-<u>O</u>-isopropylidene-β-
<u>D</u>-fructopyranoses.
  R. F. Brady, Jr., Carbohyd. Res. <u>15</u>, 35 (1970).

Electron-spin Resonance Study of an Alkaline Solution
of Copper(II) Oxalate-<u>meso</u>-Tartrate Complex (the
Somogyi Reagent) and Related Complexes.
  A. J. Fatiadi, J. Res. NBS <u>74A</u>, 723 (1970).

Determination of Moisture in Starch Hydrolyzates by
Near-Infrared and Infrared Spectrophotometry.
  D. W. Vomhof and J. H. Thomas, Anal. Chem. <u>42</u>,
  1230 (1970).

Oxidative Cleavage of Aromatic Azines with Periodic Acid:
E.s.r. Evidence for the Liberation of Nitrogen Dioxide.
   A. J. Fatiadi, Chem. Ind. (London) 64 (1971).

Cyclic Acetals of Ketoses. Part IV. Re-investigation
of the Oxidation of 1,2:4,5-Di-O-isopropylidene-β-D-
fructopyranose with Methyl Sulfoxide—Acetic Anhydride.
   R. S. Tipson, R. F. Brady, Jr., and B. F. West,
   Carbohyd. Res. 16, 383 (1971).

Determination of Inososes with an Alkaline Solution of
Copper(II) Oxalate—Tartrate Complex (the Somogyi
Reagent) and Reaction Mechanisms Involved.
   A. J. Fatiadi, Carbohyd. Res. 17, 419 (1971).

C.   Manuscripts in Process of Publication

Occurence, Properties, and Preparation of Naturally
Occurring Monosaccharides, Including 6-Deoxy Sugars.
   R. Schaffer. (Manuscript accepted for "The
   Carbohydrates," edited by W. Pigman and D. Horton,
   Vol. IA, in press)

Infrared Spectroscopy of Carbohydrates.
   R. S. Tipson and F. S. Parker. (Manuscript accepted
   for "The Carbohydrates," edited by W. Pigman and
   D. Horton, Vol. IB, in press)

Conformational Analysis via Nuclear Magnetic Resonance
Spectroscopy.
   B. Coxon. (Manuscript accepted for "Methods in
   Carbohydrate Chemistry," edited by R. L. Whistler
   and J. N. BeMiller, Vol. 6, in press)

Review of "The Carbohydrates: Chemistry and
Biochemistry," 2nd edition, Volume IIA, edited by
W. Pigman and D. Horton, Academic Press, New York, 1970.
   R. S. Tipson. (Manuscript accepted for publication
   in Carbohydrate Research)

Cyclic Acetals of Ketoses.
   R. F. Brady, Jr. [Manuscript accepted for publication
   in Advances in Carbohydrate Chemistry and Biochemistry,
   26 (1971)].

Evidence for Adsorption as the First Step in the Solid-
state Oxidation of Benzenehexol with Active Manganese
Dioxide.
   A. J. Fatiadi. [Manuscript accepted for publication
   in J. Chem. Soc. (B)].

Enolization of Hexodiulose Acetals. Synthesis of
Derivatives of D-Psicose by Reduction of a Hex-3-
enulopyranose.
   R. F. Brady, Jr. (Manuscript accepted for publication
   in Carbohydrate Research)

Application of Internuclear, Double-resonance Techniques to Carbohydrates. Detection of Small Coupling-constants.
B. Coxon. (Manuscript accepted for publication in Carbohydrate Research)

(p-Bromophenyl)osotriazoles from Inositols.
A. J. Fatiadi. (Manuscript accepted for publication in Carbohydrate Research)

N.m.r. Spectroscopy of Derivatives of 6-Amino-6-deoxy-D-glucose-6-^{15}N. ^{13}C Fourier Transform and Internuclear Double- and Triple-resonance Studies.
B. Coxon and L. F. Johnson. (Manuscript accepted for publication in Carbohydrate Research)

Stable Radical-anions Derived from Glyoxal Bis(phenylhydrazones).
A. J. Fatiadi. (Manuscript accepted for publication in J. Res. NBS)

Determination of Moisture in Syrups by Gas—Solid Chromatography.
D. W. Vomhof and J. H. Thomas. (Manuscript submitted for publication in J. Ass. Offic. Anal. Chem.)

Cholesterol: Standard Reference Material 911.
R. Schaffer, A. Cohen, and R. F. Brady, Jr. (Manuscript submitted for publication in American Journal of Clinical Pathology)

Periodic Acid as a New Oxidant for the Degradation of Bile Pigments. Isolation of a Biliverdine Type of Reaction Intermediate on Oxidation of Bilirubin with Periodic Acid.
A. J. Fatiadi and R. Schaffer. (Manuscript submitted for publication in Experientia)

Characterization and Quantitative Analysis of D-Glucose for Use in Clinical Analysis.
B. Coxon and R. Schaffer. (Manuscript submitted for publication in Analytical Chemistry)

Documentation Interest of the National Bureau of Standards and Collaborating Groups for Communication of Laboratory Results.
R. Schaffer. (Manuscript submitted for publication in Proceedings of a Conference on Clinically Oriented Documentation of Laboratory Data, Buffalo, N. Y., May 10—12, 1971)

D. Lectures

9/18/70 - Programs for Standards Relating to Clinical Laboratories. National Committee for Clinical Laboratory Standards, Atlanta, Georgia.
R. Schaffer.

10/9/70 - NBS SRMs for Clinical Analysis. Symposium on
Standardization for Meaningful Clinical
Analysis, National Bureau of Standards.
R. Schaffer.

11/24/70 - Studies of Carbohydrates by Internuclear,
Magnetic Double-resonance Techniques.
Department of Chemistry, Villanova
University, Villanova, Pennsylvania. B. Coxon.

1/5/71 - Application of Internuclear Double- and
Triple-resonance Techniques to Carbohydrates.
School of Molecular Sciences, University of
Sussex, Brighton, England. B. Coxon.

2/4/71 - Enolization of Hexodiulose Derivatives.
Synthesis and Reduction of a 3,4-Enediol
Acetate. Sixth Middle Atlantic Regional
Meeting of the American Chemical Society,
Baltimore, Maryland. R. F. Brady, Jr.

4/1/71 - Studies of 6-Deoxy Sugars by Internuclear,
Magnetic Double-resonance Techniques.
American Chemical Society Meeting,
Los Angeles, California. B. Coxon.

4/15/71 - The Use of Differential Scanning Calorimetry
and Phase Solubility Analysis in the
Characterization of Clinical Standard
Reference Materials. Merck, Sharp and Dohme
Research Laboratories, Rahway, New Jersey.
R. F. Brady, Jr.

5/12/71 - Clinical Chemical Analysis Tied to NBS SRMs.
State University of New York, Buffalo,
New York. R. Schaffer.

5/16/7] - The NBS Program to Aid Clinical Chemical
Analysis. American Association of Clinical
Chemists, Wrightsville, North Carolina.
R. Schaffer.

5. ACKNOWLEDGMENTS

6. REFERENCES

[1] Recommendation on a Uniform Bilirubin Standard, Clin. Chem. 8, 405 (1962).

[2] Henry, R. J., Jacobs, S. L., Chiamori, N., Clin. Chem. 6, 529 (1960).

[3] Fog, J., Scand. J. Clin. Lab. Invest. 16, 49 (1964).

[4] With, T. K., Bile Pigments, Academic Press, Inc., New York, N. Y., 1968, pp. 22-23.

[5] Nicholson, D. C., Stoll, M., personal communication.

[6] Petryka, Z. J., Watson, C. J., J. Chromatogr. 37, 76 (1968).

[7] Fatiadi, A. J., Schaffer, R., Experientia, in press.

[8] Dybkaer, R., Hertz, H., Scand. J. Clin. Lab. Invest. 25, 151 (1970).

[9] Gadd, K. G., J. Clin. Pathol. 19, 300 (1966); Simmons, N. A., ibid. 21, 196 (1968); Billing, B. H., Halsom, R., Wald, A., Ann. Clin. Biochem., in press.

[10] Michaëlson, M., Scand. J. Clin. Lab. Invest. 13, Suppl. 56, 46 (1961).

[11] Cohen, A., NBS Technical Note 457, 77 (1968).

[12] Kammereck, R., Lee, Wen-Hui, Paliokas, A., Schroepfer, G. J., Jr., J. Lipid Res. 8, 282 (1967).

[13] Brady, R. F., Jr., NBS Technical Note 457, 70 (1968).

[14] Brady, R. F., Jr., NBS Technical Note 507, 97 (1969).

[15] Brady, R. F., Jr., NBS Technical Note 547, 24 (1970).

[16] Hayden, A. L., Anal. Chem. 27, 1486 (1955).

[17] Brady, R. F., Jr., NBS Technical Note 457, 58, 90 (1968).

[18] Parsons, W. H., Gennaro, A. R., Osol, A., Amer. J. Pharm. 133, 351 (1961).

[19] Coxon, B., NBS Technical Note 507, 28 (1969).

[20] Coxon, B., NBS Technical Note 547, 68, 75, 77 (1970).

[21] Coxon, B., Schaffer, R., Anal. Chem., in press.

[22] Gardner, J. A. F., Hibbert, H., J. Amer. Chem. Soc. 66, 607 (1944).

[23] Shaw, K. N., McMillan, A., Armstrong, M. D. J. Org. Chem. 23, 27 (1958); Recondo, E. F., Rinderknecht, H., J. Org. Chem. 25, 2248 (1960).

[24] Goodman, I., Olenczak, A. P., Scherrer, J. W., Hiatt, R. B., Biochim. Biophys. Acta 156, 364 (1968).

[25] Boedecker, F., Volk, H., U. S. Pat. 2,062,205; Kamlet, J., U. S. Pat. 2,640,083 [Chem. Abstr. 48, 5884 (1954)]

[26] Reference 21, p.38.

[27] J. LeMaistre, personal communication.

[28] Coxon, B., NBS Technical Note 457, 70 (1968).

[29] Richtmyer, N. K., Hudson, C. S., J. Amer. Chem. Soc. 73, 2249 (1951).

[30] C. J. Pouchert, Aldrich Library of Infrared Spectra, spectrum No. 93c.

[31] Specifications and Criteria for Biochemical Compounds, Tipson, R. S.(Ed.), Publication 1344, National Academy of Sciences—National Research Council, Washington, D. C., 1967, CoE-7, p. 207.

[32] Burton, R. M. Pietro, A. S., Arch. Biochem. Biophys. 48, 184 (1954).

[33] Cohen, A., NBS Technical Note 547, 47 (1970).

[34] Ciotti, M. M., Kaplan, N. O., Methods Enzymol. 3, 890 (1957).

[35] Worthington Biochemical Corp., Freehold, New Jersey.

[36] Colour Index, Society of Dyers and Colourists, Second edition, Vol. 3, 1956, p. 3513.

[37] Tipson, R. S., Brady, R. F., Jr. Carbohyd. Res. 10, 549 (1969).

[38] Brady, R. F., Jr., Carbohyd. Res. 15, 35 (1970).

[39] Tipson, R. S., Brady, R. F., Jr., West, B. F., Carbohyd. Res. 16, 383 (1971).

[40] Horton, D. Just, E. K., Carbohyd. Res. 9, 129 (1969).

[41] Barton, D. H. R., Evans, R. M., Hamlet, J. C., Jones, P. G., Walker, T., J. Chem. Soc. 747 (1954); Barton, D. H. R., Ives, D. A. J., Thomas, B. R., ibid. 903 (1954).

[42] Franchimont, M., Compt. Rend. 92, 1054 (1881).

[43] Marshall, C. W., Kritchevsky, T. H., Lieberman, S., Gallagher, T. F., J. Amer. Chem. Soc. 70, 1837 (1948).

[44] Isbell, H. S., Tipson, R. S., J. Res. Nat. Bur. Stand. 64A, 171 (1960).

[45] Takagi, S., Rosenstein, R. D., Acta Crystallogr. A25 [S3], S197 (1969); Carbohyd. Res. 11, 156 (1969).

[46] Maeda, T., Kiyokawa, M., Tokuyama, K., Bull. Chem.
Soc. Jap. 38, 332 (1965); Maeda, T., ibid. 40,
2122 (1967); Maeda, T., Tori, W., Satoh, S.,
Tokuyama, K., ibid. 42, 2635 (1969).

[47] Angyal, S. J., Macdonald, C. G., J. Chem. Soc. 686 (1957).
(1957).

[48] Levene, P. A., Tipson, R. S., J. Biol. Chem.
93, 631 (1931); Tipson, R. S., Cohen, A., Carbohyd.
Res. 7, 232 (1968).

[49] Reichstein, T., Bosshard, W., Helv. Chim. Acta 17,
753 (1934).

[50] Coxon, B., Carbohyd. Res. 12, 313 (1970); 13, 321
(1970); 14, 9 (1970).

[51] Baker, E. B., J. Chem. Phys. 37, 911 (1962).

[52] Kaiser, R., J. Chem. Phys. 39, 2435 (1963).

[53] Ferretti, J. A., Freeman, R., J. Chem. Phys. 44,
2054 (1966).

[54] Coxon, B., Carbohyd. Res., in press.

[55] Coxon, B., Carbohyd. Res., 11, 153 (1969).

[56] Pasto, D. J., Johnson, C. R., Organic Structure
Determination, Prentice-Hall, Inc., Englewood
Cliffs, N. J. (1969), pp. 110 and 137.

[57] Budzikiewicz, H., Djerassi, C., Williams, D. H.,
Mass Spectrometry of Organic Compounds, Holden-Day,
San Francisco, Calif. (1967), p. 225.

[58] Coxon, B., Carbohyd. Res., in press.

[59] Binsch, G., Lambert, J. B., Roberts, B. W., Roberts,
J. D., J. Amer. Chem. Soc. 86, 5564 (1964).

[60] Emsley, J. W., Feeney, J., Sutcliffe, L. H.,
High Resolution Nuclear Magnetic Resonance
Spectroscopy, Vol. 2, Pergamon Press, Oxford (1966),
p. 878.

[61] Coxon, B., Johnson, L. F., Carbohyd. Res., in press.

[62] Fatiadi, A. J., Carbohyd. Res., in press.

[63] Littler, J. S., University of Bristol, England,
personal communication.

[64] Woodward, R. B., Hoffmann, R., Angew. Chem. Intern.
Ed. 8, 781 (1969).

[65] Zimmerman, H. E., Angew. Chem. Intern. Ed. 8, 1
(1969); see also, Pearson, R. G., Accounts Chem.
Res. 4, 152 (1971).

[66] Littler, J. S., Tetrahedron 27, 81 (1971).

[67]   Fatiadi, A. J. Chem. Ind. (London) 64 (1970).

[68]   Kiselev, A. U., Quart. Rev. (London) 15, 99 (1961).

[69]   Evans, R. M., Quart. Rev. (London) 13, 61 (1959).

[70]   Gattow, G., Wendlandt, H. G., Z. Anal. Chem. 174, 15 (1960).

[71]   Inorganic Index to Powder Diffraction, File 1969, Amer. Soc. Testing Materials, Philadelphia, Pa., 1969, p. 701.

[72]   Ayschough, P. B., Electron Spin Resonance in Chemistry, Methuen, London, 1967, p. 170.

[73]   Fatiadi, A. J., Sager, W. F., Org. Synth. 42, 66 (1962)

[74]   Stone, F. S., Advan. Catal. 1, 13 (1952).

[75]   Dillimore, D., Tonge, K. H., J. Chem. Soc. (B) 1380 (1967).

[76]   Fatiadi, A. J., Isbell, H. S., Sager, W. F., J. Res. NBS 67A, 153 (1963).

[7]   Pratt, E. F., Van de Castle, J. E., J. Org. Chem. 26, 2973 (1961).

[78]   Konaka, J., Terabe, S., Kuruma, K., J. Org. Chem. 34, 1334 (1969).

[79]   Fatiadi, A. J., J. Chem. Soc. (B) 889 (1971).

[80]   Buchachenko, A. L., Stable Radicals, Consultants Bureau, New York, 1965, pp. 100—144.

[81]   Forrester, A. R., Hay, J. M., Thomson, R. H., Organic Chemistry of Stable Free Radicals, Academic Press, New York, 1968, pp. 137—179.

[82]   Razuvaev, G. A., Abakumov, G. A., Pestunovich, V. A., J. Struct. Chem. 5, 278 (1964).

[83]   Walter, R. J., J. Amer. Chem. Soc. 88, 1923 (1966).

[84]   Maender, O. W., Russell, G. A., J. Org. Chem. 31, 442 (1966); Russell, G. A., in Radical Ions, Kaiser, E. T., Kevan, L. (Eds.), Interscience, New York, 1968, p. 87 ff.

U.S. DEPT. OF COMM. BIBLIOGRAPHIC DATA SHEET	1. PUBLICATION OR REPORT NO. NBS TN-587	2. Gov't Accession No.	3. Recipient's Accession No.

4. TITLE AND SUBTITLE	5. Publication Date
Organic Chemistry Section: Summary of Activities, July 1970 to June 1971	August 1972
	6. Performing Organization Code

7. AUTHOR(S) Dr. Robert Schaffer, Editor	8. Performing Organization

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. Project/Task/Work Unit No.
· NATIONAL BUREAU OF STANDARDS DEPARTMENT OF COMMERCE WASHINGTON, D.C. 20234	11. Contract/Grant No.

12. Sponsoring Organization Name and Address	13. Type of Report & Period Covered Interim - July 1970 to June 1971
Same as No. 9.	14. Sponsoring Agency Code

15. SUPPLEMENTARY NOTES

16. ABSTRACT (A 200-word or less factual summary of most significant information. If document includes a significant bibliography or literature survey, mention it here.)

This report of the Organic Chemistry Section of the National Bureau of Standard's Institute for Materials Research provides a summary of a year's scientific activity; as such, it covers both work that was completed and some that is still in progress. The development of Standard Reference Materials (SRM's) and their certification, and journal publications describing aspects of that work, as well as other research activities, are the output of the Section.

In the SRM category, the major subjects of this report are bilirubin, cholesterol, cortisol, $\underline{D}$-glucose, 4-hydroxy-3-methoxy-$\underline{DL}$-mandelic acid (VMA), $\underline{D}$-mannitol, and the reduced form of nicotinamide adenine dinucleotide (NADH).

Studies concerning carbohydrates include synthetic work with $\underline{D}$-psicose, n.m.r. employing internuclear double-resonance, and mass spectroscopy of 6-deoxy-1,2:3,5-di-$\underline{O}$-isopropylidene-α-$\underline{D}$-glucose, spectroscopic studies of derivatives of 6-amino-6-deoxy-$\underline{D}$-glucose-6-^{15}N (employing mass spectrometry, Fourier-transform, ^{13}C-n.m.r. and heteronuclear, ^{15}N indor techniques), and $\underline{p}$-bromophenylosotriazoles of inositols.

Other research utilized e.s.r. and involved periodic acid oxidation of azines, solid-state oxidation of benzenehexol on active manganese dioxide, and the formation of stable radical-anions from glyoxal bis(phenylhydrazone).

17. KEY WORDS (Alphabetical order, separated by semicolons)

Bilirubin; cholesterol; cortisol; e.s.r.; $\underline{D}$-mannitol; NADH; n.m.r.; purity; Standard Reference Materials; SRM; VMA.

18. AVAILABILITY STATEMENT	19. SECURITY CLASS (THIS REPORT)	21. NO. OF PAGES
[X] UNLIMITED.	UNCLASSIFIED	131
[ ] FOR OFFICIAL DISTRIBUTION. DO NOT RELEASE TO NTIS.	20. SECURITY CLASS (THIS PAGE) UNCLASSIFIED	22. Price $1.25